DEVELOPMENT OF AN ONLINE MICRODIALYSIS-MICROCHIP ELECTROPHORESIS SYSTEM FOR NEAR REAL TIME MONITORING OF AMINO ACIDS

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This thesis is dedicated to my parents, Pankaj K. Desai and Meenaxi P. Desai, wonderful loving husband, Ankit Parikh, caring siblings, Naiya P. Desai and Deep Desai, in-laws, Rohit Parikh and Nayana Parikh, grand parents, professors and friends who have encouraged and supported me in pursing towards this path.

ABSTRACT

The brain extracellular space is complex chemical environment consisting of ions, peptides, neurotransmitters, growth factors, metabolites and extracellular matrix molecules. Microdialysis is a popular and well-characterized sampling technique that makes it possible to continuously recover analytes from the extracellular space. Systems that couple microdialysis with capillary electrophoresis (CE) based separations have gained popularity due to their ability to perform online analysis with high temporal resolution. Such systems are appropriate to study *in vivo* neurotransmission where the fluctuations in neuroactive analytes can occur in the second to millisecond time scale. In this work, a separation based microdialysis-microchip electrophoresis system with fluorescence detection was developed for the determination of amino acid neurotransmitters from microdialysis samples collected from rat brain striatum. Amino acid standards and microdialysis samples were derivatized with naphthalene 2, 3 dicarboxaldehyde (NDA) and sodium cyanide (NaCN) to form fluorescent cyno-2-substituted-benz(f) isoidole (CBI) derivatives prior to analysis.

Initial experiments employed capillary electrophoresis with laser induced fluorescence (CE-LIF) detection to evaluate the optimum buffer conditions for the separation of amino acid standards. This was followed up with the analysis of a rat brain microdialysate sample by CE-LIF in which aspartate (Asp) and glutamate (Glu)

were the primary amino acids of interest. Lastly, microdialysis samples were analyzed for Glu release *in vivo* triggered by a high potassium infusion.

The CE separation of amino acids served as the basis for optimization of the amino acid separation in the microchip electrophoresis experiments. First, offline analysis was carried out on brain microdialysate samples to optimize the method for the detection of Asp and Glu. The optimal separation conditions consisted of 200 mM borate, 80 mM SDS, 5% acetonitrile using a 21 cm long serpentine channel in a PDMS microchip. Subsequently, the chip was modified for online monitoring with precolumn derivatization by introducing three separate tubing inlets for microdialysis sample and reagents introduction followed by mixing in a serpentine reaction channel. The derivatized sample was then injected, separated and detected by microchip electrophoresis with fluorescence detection. This device was successfully employed for the continuous monitoring of Asp, Glu and fluorescein in the striatum of an anesthetized Sprague-Dawley (SD) rat. Fluorescein was also included in the separation for the possibility of monitoring blood brain barrier (BBB) integrity simultaneously with amino acid neurotransmitter release.

A possible application of this device is monitoring changes in excitatory amino acids and BBB integrity due to stroke. During stroke, the concentration of Glu is increased leading to excitotoxicity and loss of integrity of the BBB. Our work established an online microdialysis microchip setup that can be further modified to

perform high temporal resolution studies for studying dynamic changes of neuroactive analytes in the brain following stroke.

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LIST OF ABBREVATIONS

Glu	Glutamate
Asp	Aspartate
CNS	Central nervous system
LC	Liquid chromatography
CE	Capillary electrophoresis
MS	Mass spectrometry
BBB	Blood Brain Barrier
aCSF	Artificial cerebral spinal fluid
LIF	Laser induced fluorescence
BGE	Background electrolyte
EOF	Elctroosmotic flow
CZE	Capillary zone electrophoresis
MEKC	Micellar electrokinetic capillary chromatography
SDS	Sodium dodecyl sulfate
CMC	Critical micelle concentration
NDA	Naphthalene 2, 3 dicarboxaldehyde
CN^-	Cyanide
SD	Sprague-Dawley
CBI	Cyno-2-substituted-benz(f) isoidole
MCE	Microchip electrophoresis
PDMS	(Poly)dimethylsiloxane

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Introduction

L-Glutamate (Glu) aspartate (Asp) are major excitatory neurotransmitter in the central nervous system (CNS). These excitatory amino acids are also engaged in intermediary metabolism in neural tissue and play an important role in detoxification of ammonia, long term potentiation, and the synthesis of proteins and peptides. The concentration of Glu is much higher in the CNS than in other tissues. Imbalance of excitatory neurotransmitters in the extracellular space (particularly Glu) has been implicated in various neurological disorders such as epilepsy, schizophrenia and stroke where Glu can exert strong excitotoxic effects [1] . Excitotoxicity is defined as an overactivity of a specific receptor, where it damages surrounding cells. For example, during stroke release of Glu leads to release of more Glu and activation of adjoining NMDA receptors. Also in the CNS, Glu concentration is of therapeutic interest for the development of drugs to reduce brain damage following head injury or stroke [2]. Therefore, development of an analytical method for monitoring amino acid neurotransmitters in the CNS is of paramount importance. In addition, near real-time monitoring of such compounds by suitable techniques can provide a "live" picture of changes in the concentration of neurotransmitters in the CNS during rapid brain attack such as stroke.

Microdialysis sampling is an effective and popular technique for small molecule analyte sampling from the CNS. This procedure can be employed to collect

samples for offline analysis by liquid chromatography (LC), CE, mass spectrometry (MS) as well as other techniques. For online monitoring, microdialysis can be coupled with CE or LC to continuously inject and analyze samples at a reasonably fast rate yielding "near real-time" data. Over the past few years, our group has been involved in development of miniaturized CE systems (microchips) for online continuous analysis of microdialysis samples *in vitro*. In this thesis, efforts were directed towards the development and characterization of microdialysis-microchip systems for *in vivo* analysis. The rat stroke model was considered to be one target application of such a system. The ultimate goal of this work is to apply the optimized online microdialysis-microchip system to monitor Glu release, BBB permeability, and changes in excitatory amino acids during stroke.

1.2 Stroke

Stroke is the third leading cause of death in United States [12]. According to 2009 statistics, the total economic burden in the U.S. due to stroke related health problems is approximately $165.4 billion per year [3]. Hypertension, diabetes, obesity, smoking, high cholesterol, atrial fibrillation, sickle cell anemia and age increases predisposition to stroke. In United States, stroke death rates are higher in the southeastern part compared to other regions indicating geographical impacts on stroke mortality. Also, statistics suggest that stroke related mortality rates are higher in women than in men. The risk factor of stroke is 22 times higher in women over the

age of 30 who smoke and take high estrogen oral contraceptives. Seventy-five percent of stroke survivors end up with some type of disability (e.g. difficult in speaking and vision, unexpected confusion, emotional difficulties and weakness or paralysis of a body part).

Stroke can be thought of as a "brain attack" resulting from blockage (ischemic stroke) and/or rupture of blood vessels (hemorrhagic stroke) in the brain that results in the interruption of blood flow to specific areas of the brain. Ischemic stroke can occur due to atherosclerosis, thrombosis, embolism or systemic hypoperfusion and is the causative factor in 85% of stroke incidents [4]. Hemorrhagic stroke affects approximately 15% of stroke patients. Better treatment is available for victims of ischemic stroke in terms of medications (antiplatelet and anticoagulant drugs) and preventive aspects (cholesterol medications, active lifestyle and heart-healthy diet). This leads to better chances of survival of ischemic stroke victims over hemorrhagic stroke.

1.3 Stroke and excitatory amino acids

Glu and Asp occur in high concentrations in the brain and are known to exert powerful stimulatory effects on neuronal activity [5]. To avoid neuronal damage, the concentration of Glu is tightly regulated in the extracellular space and is kept under firm control by Glu transporters that are present in the plasma membranes of the

nerve cells [6]. It has been shown that Glu exhibits excitotoxicity effects as a consequence of its accumulation in the extracellular space following long-term stimulation of post synaptic Glu receptors (i.e. the NMDA receptors) [7,8]. During ischemic stroke, reduced oxygen levels disrupt energy (ATP) production, which is necessary for the survival of any tissue. This results in membrane depolarization and release of Glu into the extracellular space [9]. Glu binds to nearby NMDA receptors thereby activating them. This subsequently leads to a surge in the influx of calcium ions (Ca^{+2}) hyperpolarizing the cells and overloading them with calcium ions [10]. Calcium ions activate many enzymes including those causing digestion of cellular organelles. It also damages mitochondria and shuts them down causing further energy depletion and ultimately resulting in programmed cell death or apoptosis as shown in Figure 1 [11]. This pathway of excess Glu induced nerve cell damage followed by cell death is referred to as excitotoxicity.

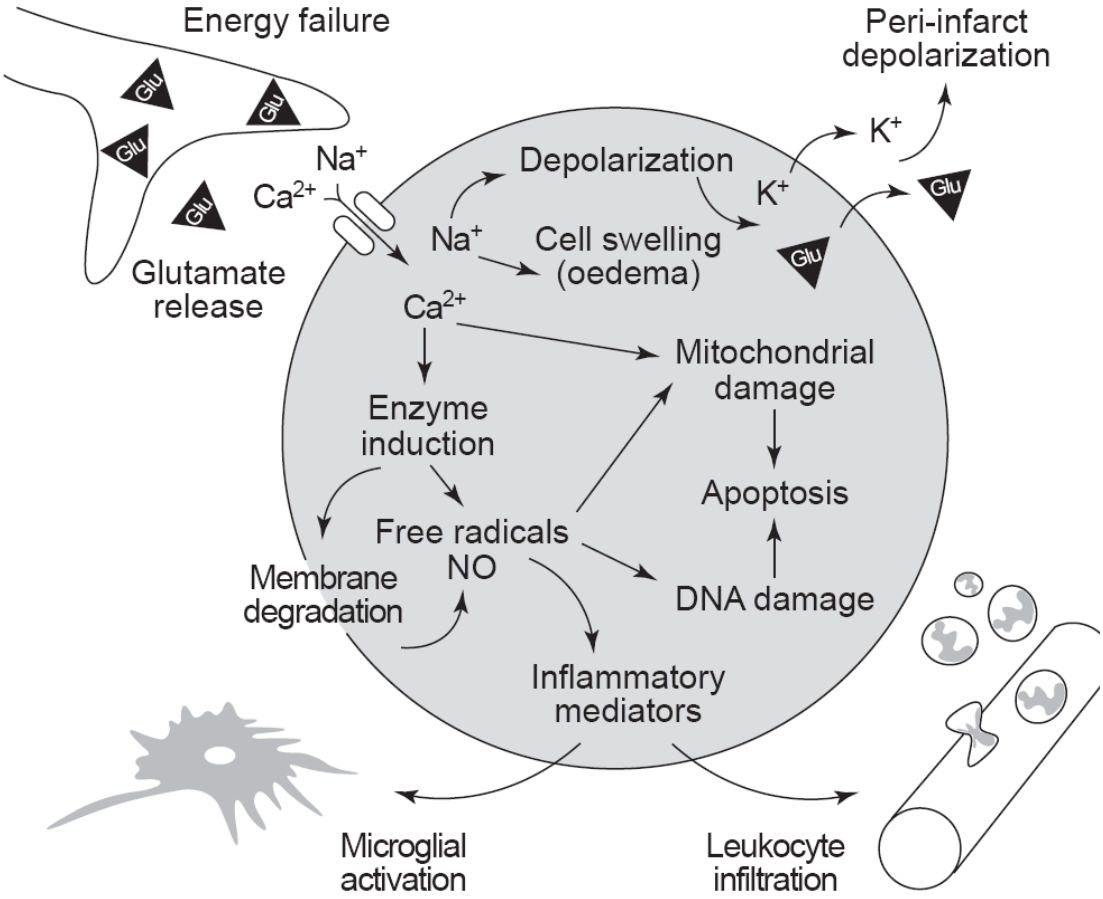

Figure 1: Illustrates event of stroke trigged due to the excitotoxicity of Glu in the brain. Ischemic stroke occurs due to reduction of oxygen level causing membrane depolarization thus release of Glu in extracellular space. Binding of Glu to NMDA receptors causes influx of Ca^{+2}. This leads to hyperpolarization of cells and ultimately cell death. [12].

The blood brain barrier (BBB) is an assembly of endothelial cells located at the level of brain capillaries [11,12]. The cells in this barrier are joined together by tight junctions. The BBB helps to maintain a "stable" environment in the brain by making it inaccessible to many compounds that can produce potentially toxic effects in the CNS. Stroke-induced Glu excitotoxicity disrupts the tight junctions of the BBB and renders it "leaky" resulting in exposure of brain cells to harmful compounds [15,16].

1.4 Microdialysis sampling

1.4.1 Set-up

Microdialysis is a sampling technique that allows the continuous retrieval of analytes from the extracellular fluid of the brain and other tissues. The power of this method lies in the fact that samples can be collected from awake freely moving animals. To perform microdialysis experiments, a probe is surgically implanted into the target tissue. The probe is perfused with a solution that is isotonically comparable to the extracellular fluid of the tissue. Brain microdialysis probes typically consists of a dialysis membrane (2 - 4 mm length) located at the end of a stainless steel cylindrical concentric cannula, the inner diameter of which varies between 200 – 500 μm. Other types of microdialysis probes include linear probes for soft tissue sampling, flexible probes for blood sampling and shunt probes for bile sampling [17].

In the case of brain probes, the isotonic salt solution artificial cerebral fluid (aCSF) which is the perfusate pumped through the microdialysis inlet following probe implantation. The perfusate passes from the inner tubing to the outer semipermeable tubing at which point, exchange of analytes occurs between the probe and surrounding matrix. Small molecules diffuse in and out across the membrane based on their concentration gradient without any net fluid loss. The sample is collected at the outlet of the probe for further analysis as shown in Figure 2.

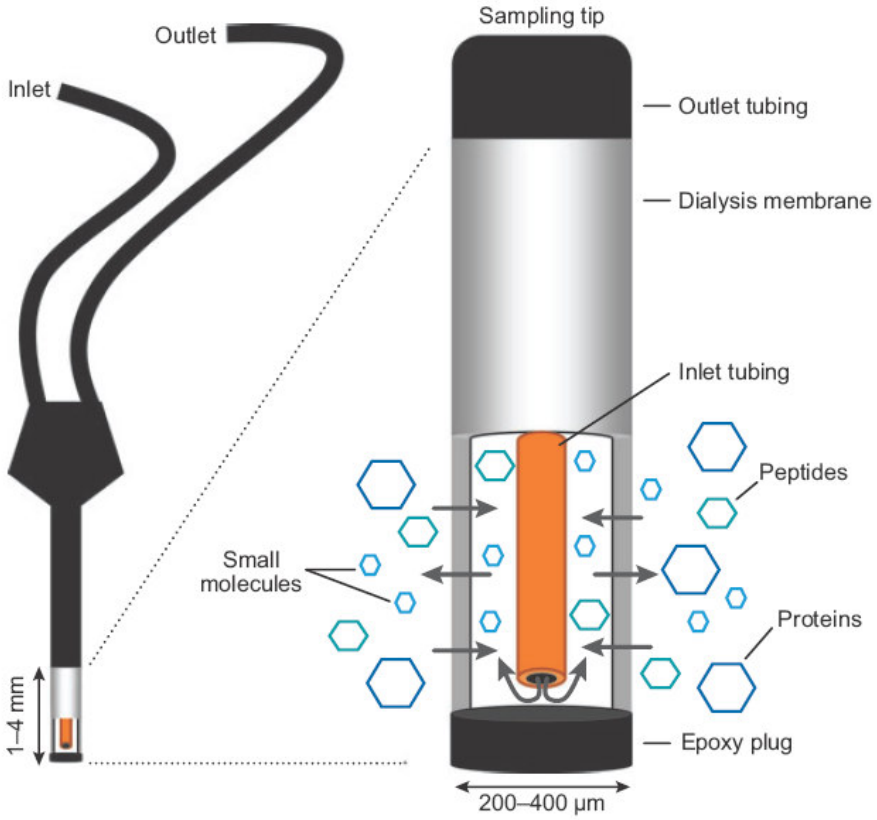

Figure 2: Microdialysis probe used for sampling from rat brain striatum [10]

1.4.2 Recovery

Recovery is an important parameter for microdialysis to accurately measure the concentration of *in vivo* analytes. The problem associated with estimating recovery is mainly affected by the fact that the analyte is continuously carried away by the perfusion medium and therefore can never be at equilibrium with the external environment. Other factors that affect recovery are type and length of membrane, surface area and molecular weight cutoff of the probe, geometry of the probe, sample matrix, physical properties of the analyte and perfusion flow rate. Other considerations include loss of tissue integrity after surgery, changes in blood flow and immunological reactions after insertion of the probe [19].

The microdialysis probe recovery is calculated based on the equation: $R_c = C_d/C_s$; where R_c is the recovered analyte concentration, C_d is the dialysate analyte concentration and C_s is the original sample concentration. When a microdialysis probe is placed in a medium of analyte *in vivo* (i.e. brain extracellular space), the analyte is transported via three steps which happen in series. The first step is analyte transport through the media (tissue), then diffusion across microdialysis membrane takes place followed by dispersion of the analyte in the microdialysate. Taking all factors into consideration, an alternative equation for recovery (R_c) can be expressed as:

$$R_c = 1 - \exp(-1/(Q_d(R_d + R_m + R_e))$$

Where R_d, R_m and R_e are the resistances to diffusion of the dialysate, membrane and medium external to the probe respectively [20].

Recovery (R_c) in microdialysis is exponentialy related to the volumetric flow rate (Q_d) of the perfusate through the probe. Lower flow rates generate higher recoveries because there is more time to diffuse in and out through the probe. However, the major disadvantage of low flow rates is that less volume of sample is collected over time. This can be problematic for analysis especially with online systems seeking to attain high temporal resolution (discussed in Chapter 3). The flow rates used for sampling generally range between 0.5-2.0 μL/min.

The perfusion fluid is closely matched in ionic strength and composition to the tissue interstitial fluid so that there is no net osmotic fluid loss in either direction. Recovery has been reported to be affected by difference in tonicity [17]. As most *in vitro* studies involve stirred systems (making R_e negligible), it is important to consider the analyte property as the slowest mass transfer step for the analyte to diffuse through the tissue. The slow diffusion of analyte through extracellular fluid can affect recovery and calculations based on *in vitro* recovery values can produce erroneous estimates. The variability of diffusion coefficients (based on physicochemical property of the analyte) needs to be borne in mind as a small molecule (amino acid neurotransmitter) will possibly diffuse much faster than a larger molecule (peptide neuromodulators) [20].

In order to obtain reliable data from microdialysis sample, calibration of the probe is absolutely necessary. The traditional method of choice is "no-net flux method" for *in vivo* calibration during steady state [21]. The method involves first estimating the analyte concentration in the extracellular fluid. Next, analyte is infused through the probe at concentrations above and below the estimated concentration. When the perfusate analyte concentration is higher and lower than the surrounding matrix, analyte diffuses out of and into the probe respectively. When the dialysate analyte concentration equals the external matrix concentration, a "no-net" flux point is setup where there should be no change in dialysate concentration. Following the equation [20],

$$R_c = (C_{outlet} - C_{inlet})/(C\infty_{ECF} - C_{inlet})$$

Where R_c is extraction fraction, C represents the concentration at outlet, inlet and the extracellular fluid (ECF), $(C_{outlet} - C_{inlet})$ is plotted against C_{inlet} where C_{ECF} is the Y-intercept and R_c is slope calculated from the regression line. This method of calibration is very time consuming because one has to wait to obtain the steady state conditions. Other steady state calibration methods include *in vitro* calibration, mass transfer coefficient, internal standards, *in vivo* delivery and slow perfusion method [13].

Retrodialysis or "reverse dialysis" has been described in the literature as a "more reproducible" and "less time-consuming" technique for probe calibration in clinical applications to study pharmacokinetics [21]. The underlying principle of this

method is that the rate of diffusion across the probe semipermeable membrane is the same in both directions. Therefore a reference compound (internal standard) that closely resembles the compound of interest is added to the perfusate and the rate of disappearance of the same is calculated following the equation [20]:

Recovery (%) = $100 - (100 \times C_{dialysate}/C_{perfusate})$.

Retrodialysis calibration is performed before the experiment and the internal standard compound should be washed out by proper perfusion of the probe after calibration [22].

1.5 Online microdialysis

Microdialysis is an ideal sampling tool for coupling to online analytical systems. The primary reason is the ability of this technique to yield relatively clean samples (free of proteins and large peptides) minimizing sample preparation steps before analysis. This is achieved by the molecular weight cutoff properties of the semipermeable membrane. Also, for coupling to microfluidic devices, the low flow rate (low microliter/min to nanoliter/min) of the perfusate allows relatively easy integration (less volume of fluid that needs to be handled on chip) with possibilities to achieve better sensitivity (higher analyte recovery from the probe at low flow rates).

As microdialysis removes samples from the extracellular matrix constantly, there is greater potential to extract "time resolved" analyte concentration data if plugs can be injected and analyzed from an unbroken stream of microdialysate as frequently as possible. CE offers the capability to analyze sample in such a manner and hence is the most popular separation based analytical technique for online microdialysis systems (discussed later in chapter 2). The temporal resolution (minimum time interval necessary between repeated sample injection and separation) is an important consideration in the development of online systems as discussed later in Chapter 3.

1.6 Objectives

As mentioned earlier in this chapter, ischemic stroke triggers the release of high concentrations of Glu in the extracellular space that can eventually compromise the integrity of the BBB. However, the time course of events starting from oxygen deprivation followed by Glu excitotoxicity to loss of BBB permeability has not been explicitly studied in the literature. The objective of this work is to design and optimize an online microdialysis-microchip system for continuous real-time analysis of brain microdialysis samples. Separate optimization experiments were performed with capillary electrophoresis and then transferred to the chip based system. Online analysis was performed on chip using electrophoresis with fluorescence detection. Brain extracellular Glu was monitored along with fluorescein (injected intravenously) which served as the marker for passive permeability of the BBB (discussed later in

Chapter 3). Such proof of concept experiments demonstrate the possible application of this system for simultaneous investigation of BBB permeability and release of excitatory neurotransmitters during stroke.

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CHAPTER 2

OPTIMIZATION OF THE SEPARATION OF BRAIN AMINO ACIDS USING CAPILLARY ELECTROPHORESIS (CE) WITH LASER INDUCED FLUORESCENCE (LIF) DETECTION

2.1 Introduction of CE

The brain extracellular space is a complex chemical matrix consisting of ions, neurotransmitters, peptides, and various metabolites. The ability to analyze (separate and quantify) such neurochemically active species is of tremendous importance for the investigation of the status of various biochemical pathways in patients with CNS disorders. Such investigations can be quite complicated and challenging as most neurochemically noteworthy molecules are present in low concentrations (nanomolar) and detection of such analytes *in vivo* can be problematic. Also, the existence of many of these compounds can be quite transient (e.g. nitric oxide). However, it has been well documented that the major amino acid neurotransmitters (glutamate, aspartate, GABA, glycine) are present in relatively high concentrations (micromolar) in the CNS [1]. Therefore, analysis of amino acid neurotransmitters in the CNS can be approached as an ideal scenario to build novel analytical devices for *in vivo* monitoring without having to deal with complicated method development for the detection of unstable species, such as nitric oxide, or compounds present in trace concentrations, such as catecholamines and neuropeptides.

Capillary electrophoresis (CE) is a popular analytical technique employed for the separation and analysis of charged compounds. Presently, CE is applied for a myriad of analytical problems/methods such as genetic analysis, chiral separations, drug discovery, protein characterization, and carbohydrate analysis [2]. CE has

certain advantages over traditional separation methods such as liquid chromatography. First, separation in CE is performed in a narrow bore capillary that offers its own set of unique benefits. Considering the internal diameter (i.d.) ranging between 20-100 μm and total capillary lengths ranging from 20-100 cm, the volume of buffer required to fill the capillary for separation is a few milliliters. Also, the volume of sample required is generally considered to be 1-5% of the capillary volume; hence, the required sample volume in the capillary is in the range of nanoliters. Repeated CE analysis can be performed with few microliters of sample. The high surface to volume ratio of narrow bore capillaries also helps in effective dissipation of heat arising out of Joule heating phenomena associated with application of very high voltage across a column of material (separation buffer) [3].

A typical CE instrument consists of a narrow bore fused-silica capillary, the ends of which are immersed in the buffer vials, a high voltage power supply for voltage application across the capillary through platinum electrodes, sample and buffer vials, detection system (UV, LIF, MS), and a data collector/computer as shown in Figure 3. The first step in CE separation is filling the capillary with a suitable background electrolyte (BGE) that serves as the matrix for separation. Sample introduction into the capillary is the next step in which injection can be achieved either by pressure, leading to a sample plug being pushed into the capillary, or electrokinetic injection, where the sample plug is taken into the capillary by applying injection voltage across the capillary. This is followed by the electrophoresis

separation step where analytes are separated based on a combination of electrophoretic mobility and electroosmotic flow (EOF).

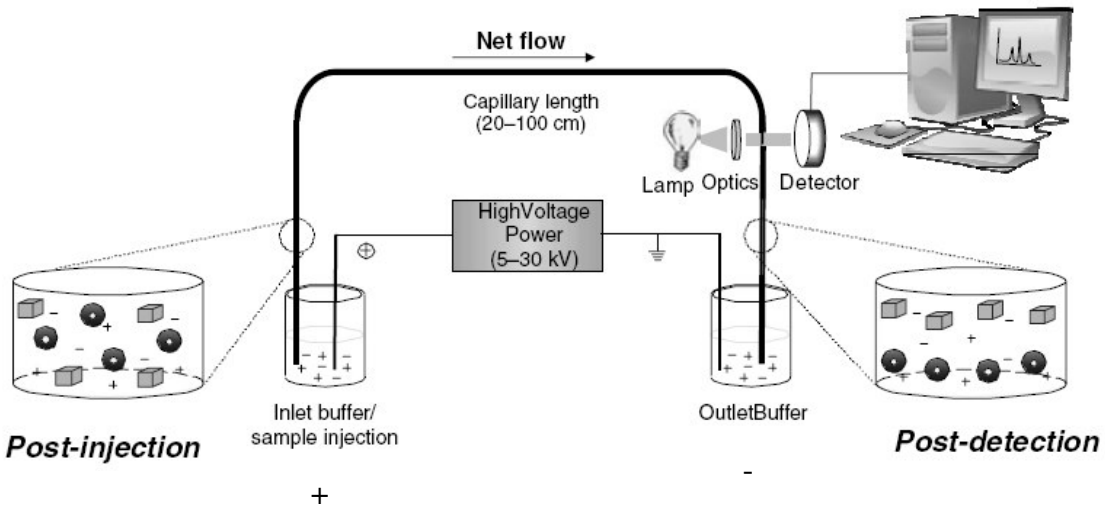

Figure 3: Basic CE instrument setup [3]

The velocity of ion or solute through a buffer solution is calculated by electrophoretic mobility (μ). The equation for representing electrophoretic mobility is:

$$\mu = q / 6\pi\eta r$$

Where q is the charge on the ion, η is viscosity of the solution and r is ion radius. Therefore μ is proportional to the charge of the ion and inversely proportional to friction coefficient. The friction coefficient (f) is dependent on size and shape of moving ion, and is defined as: $f = 6\pi\eta r$. However, the fact that analytes get driven past the detector indicates the presence of another force, i.e. the electroosmotic flow (EOF). If electrophoretic mobility was the only force, then anionic species would never migrate towards the cathode [2].

EOF is flow of the buffer generated within the capillary as shown in Figure 4a. Such bulk flow is created by the ionization of free silanol groups (depending on buffer pH) present on the internal surface of the fused silica capillary giving rise to a layer of negative charge along the wall. Therefore, the positively charged ions from run buffer are attracted to the negatively charged wall that ultimately leads to the formation of electrical double layer in order to maintain the charge balance. The double layer that is formed closely associated to the capillary wall is known as the Inner Helmholtz layer and the diffuse layer distal to the capillary wall is known as Outer Helmholtz layer. During separation, the positively charged analytes aided by

the combined effect of electrophoretic mobility and EOF (acting in same direction) elute first, followed by neutrals migrating with EOF and then negatively charged analytes as electrophoretic mobility and EOF for these ions act in opposite direction with the EOF being the stronger force. Ultimately, all the analytes are swept towards the cathode in zones as shown in Figure 4b. Maintenance of an optimal EOF is a key factor for CE analysis. High EOF may result in less time for separation of analytes leading to poorly resolved peaks. Low EOF can produce band broadening and increases the analysis time for slow migrating analytes. The apparent electrophoretic mobility of solute (μ_a) can be calculated based on a following equation: $\mu_a = \mu_e + \mu_{EOF}$. Where μ_e is defined as electrophoretic mobility and μ_{EOF} is EOF mobility. EOF is equal to $\varepsilon\zeta/4\pi\eta$; where ε is the dielectric constant, ζ is the zeta potential, and η is viscosity of the buffer [2].

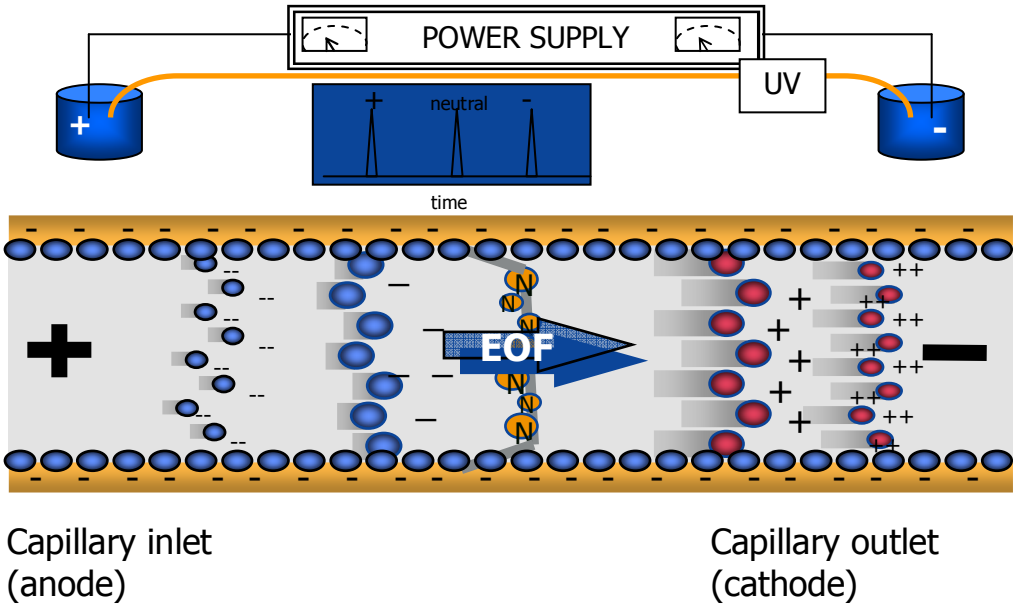

Figure 4a: Electroosmotic flow (EOF) and electrophoresis within a capillary. Capillary wall is negatively charged; therefore it attracts positive ions from a buffer forming electrical double layer. When a voltage is applied across the capillary, there is creation of bulk flow towards the cathode referred to as EOF. Analytes are then separated based on a combination of their electrophoretic mobility and electroosmotic flow.

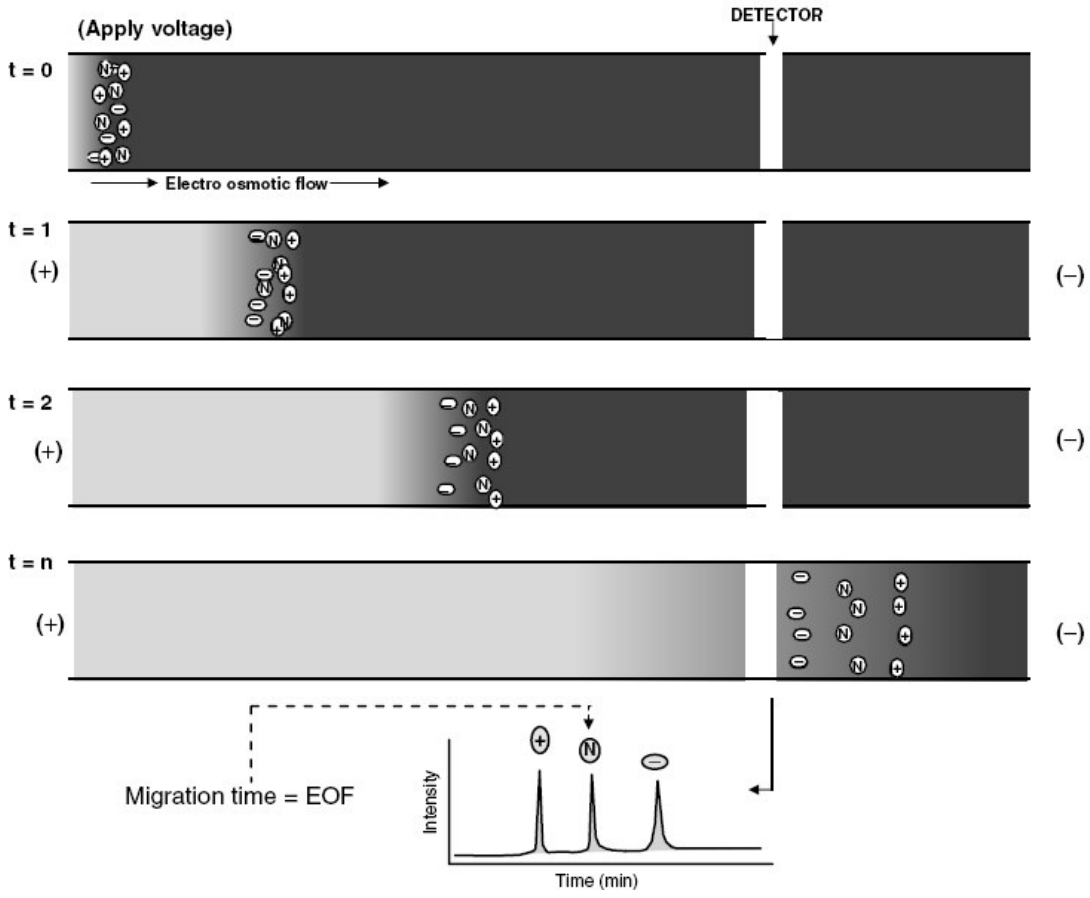

Figure 4b: Migration order of analytes in CE; the mobility due to the applied electrical potential (μ_{ep}) is towards cathode, the mobility due to the EOF (μ_{eo}) is towards anode allowing positively charge analytes to elute first followed by neutral and lastly negatively charge analytes [3].

Capillary electrophoresis has many diverse modes of separation. These include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), isotachophoresis (ITP), electrokinetic chromatography (EKC), micellar electrokinetic capillary chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), non-aqueous capillary electrophoresis (NACE), and capillary electrochromatography (CEC). In this study, MEKC analysis was performed for the separation of the derivatized amino acids.

MEKC was first reported by Terabe et. al. where he suggested the use of surfactant in the BGE to separate neutral species [4]. When a charged surfactant (e.g. sodium dodecyl sulfate (SDS) is incorporated in an aqueous buffer at a concentration greater than critical micelle concentration (CMC), micelle formation occurs in the run buffer as shown in Figure 5. Such micelles generate a pseudo-stationary phase. A neutral molecule will gain an apparent negative electrophoretic mobility as it will be incorporated in the hydrophobic core of the micelle. Also, analytes will partition in and out of the hydrophobic interior and aqueous exterior of the micelle, ultimately reaching distribution equilibrium. The migration order in MEKC is dependent on the electrophoretic mobility of the micelle, as well as the strength and the distribution coefficient of the analyte [4]. The migration velocity of neutral species is somewhere between the EOF velocity and the micelle mobility and is dependent on the extent of incorporation of the analyte with the micelle. In the cases when the EOF is stronger,

all the analytes incorporated with micelles to different extent are swept towards the cathode [5].

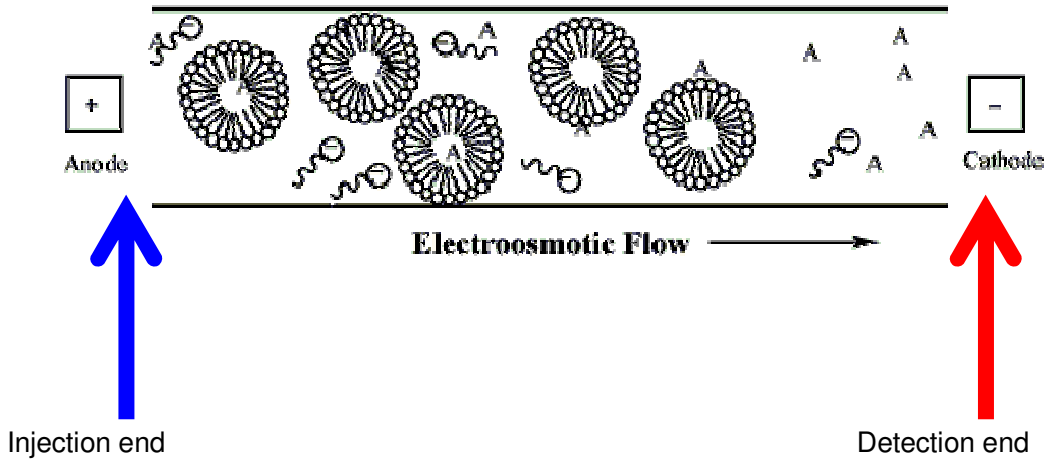

Figure 5: Migration of analytes in MEKC [6].

The advantages offered by capillary electrophoresis in terms of small volume requirement, fast analysis, efficient separation and easy integration with different detectors makes CE a well-matched technique to analyze microdialysis samples. Also, one can overcome the problems associated with the manipulation of very small volume microdialysis sample (1-20 μL) by assembling an online microdialysis CE analysis system. Sample is collected, manipulated and injected into the CE system in a continuous and efficient manner. This minimizes errors associated with manual sample handling such as sample loss, mislabeling, evaporation/degradation due to exposure from collection vials [7].

An important consideration for an online system is the frequency of sample injection and analysis to yield high temporal resolution. For microdialysis sampling, low flow rates lead to better recovery. The low flow rate creates delay between injections thus affecting the temporal resolution. On the other hand, if a high flow rate is used, there will be enough volume of sample for quick back-to-back injections (increased temporal resolution) but it will contain less analyte mass as the recovery is less. In this case, the sensitivity and selectivity of the detector becomes important. This is the reason for the popularity of fluorescence detection for online systems because low concentration of analytes in sample plugs can be detected and analyzed. Other kinds of detectors that have been used for online microdialysis CE include electrochemical and UV. Another important factor for online systems is the incorporation of an injection valve or interface that is capable of injecting plugs of

sample from the continuous stream of microdialysate. Automated injection valves containing sample loops are used for this purpose and are generally coupled between the microdialysis tubing and the separation capillary [7].

Hogan et. al. reported the first online microdialysis CE system (with LIF detection). This system was used to monitor the pharmacokinetics of an antineoplastic agent and its major metabolite in blood. The compounds were separated by MEKC mode and a separation time of 90 sec was achieved by this system [8]. Later, Zhou et. al. performed online monitoring of brain Asp and Glu following online derivatization with NDA/CN with a temporal resolution of 70 sec [9]. Later, an online system with electrochemical detector was used to monitor the transdermal delivery of nicotine over 21 hours. The temporal resolution obtained with this system was 10 minutes because of the analysis time which is a limiting factor for online systems [10].

Kennedy's group has reported multiple papers developing systems that coupled microdialysis CE with laser induced fluoresced (LIF) detection. In these cases, flow gated interface is used to inject microdialysis samples into the separation capillary. The interface was able to effectively carry out sample injection from a submicroliter/min microdialysis perfusion rate and achieve analysis time of 65–85 sec [11] . Very fast separations of Glu and Asp with a temporal resolution of 12 sec were reported in a separate paper using a short capillary (6.5cm) [12]. Quantitative

analysis was performed for multiple brain neurotransmitters using MEKC with a temporal resolution of 45 sec [13]. To improve the sensitivity by 15 fold, the group has reported the incorporation of a sheath flow cuvette. Brain amino acid monitoring was performed with this setup and 17 amino acids were separated in 30 sec following precolumn derivatization with NDA and CN^- [14].

The goal of this chapter was to develop MEKC based methods for the separation of amino acid standards as well as to identify Glu and Asp in microdialysis samples. These studies served to optimize the separation using conventional CE so that peaks of interest can be identified on the microchip setup under identical buffer conditions without any ambiguity during the development of online microdialysis-microchip CE (discussed in Chapter 3).

2.2 Experimental

2.2.1 Chemicals

The following chemicals and materials were used: boric acid, SDS, acetonitrile, disodium fluorescein, amino acid standards, sodium cyanide, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate, and disodium phosphate were purchased from Sigma (St. Louis, MO). Naphthalene 2,3 dicarboxaldehyde was purchased from Molecular Probes (Eugene,

OR). All aqueous buffers and solutions were prepared using Milli-Q water and filtered using 0.22 um Cameo Teflon syringe cartridges from Osmonics (Minnetonka, MN).

2.2.2 Microdialysis sampling and surgical procedures

A male Sprague-Dawley (SD) rat (225-250g) was initially anesthetized by inhalation of isoflurane followed by a subcutaneous (s.c.) injection containing a mixture of acepromazine (0.37 mg/mL), ketamine (37.5 mg/mL), xylazine (1.9 mg/mL). Constant body temperature was maintained by placing the animal on a heating pad. The area from the base of the skull to approximately the front of the eyes and the hair on the lower abdomen and inner thigh were shaved as closely as possible.

The animal was placed on its back for surgical insertion of femoral cannula. A small incision was made on the thigh skin with a scalpel, and the muscle was carefully separated to isolate the femoral vein from fat tissue and other arteries and veins. Once the femoral vein was clearly visualized and separated, it was looped loosely with piece of suture and a spatula was placed underneath. A small nick was made in the vein and a micro-renethane (MRE-32) cannula was inserted with aid of dental pick. The suture was tied at two different places to hold the cannula in place. The incision was closed properly with staples.

The animal was then placed on its stomach (facing surgery personnel) in the sterotaxic apparatus for implantation of microdialysis probe. An incision was made in the scalp along the mid-line and the skin was gently worked toward the sides. Small clips were then placed on both sides to hold the skin away from the mid-line. The underlying tissues were cleared to visually see the skull. Using stereotaxic coordinates A/P (+0.2), M/L (+3.2), D/V (7.5), the position for the probe was marked. Two small marks were made near the position of the probe followed by drilling 3 holes using a Dremel tool. The guide cannula was implanted in the hole designated for the microdialysis probe. Also, a bone screw was screwed in each drilled hole nearby the microdialysis probe to hold the dental acrylic. Dental acrylic was then applied to the exposed brain to anchor the guide cannula in place.

The animal was allowed to recover for 2 hours postsurgery after which the microdialysis probe (CMA 12, North Chelmsford, MA) was carefully inserted through the guide cannula. The inlet of microdialysis probe was connected via FEP tubing to a 1 mL syringe containing artifical cerebral spinal fluid (aCSF). The aCSF was composed of 120 mM of NaCl, 3 mM KCl, 20 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.25 mM Na_2HPO_4. The microdialysis probe was perfused with aCSF at flow rate of 1 μL/min (using a CMA syringe pump) for 5-10 minutes for equilibration subsequent to which samples were collected in PCR tubes for offline experiments. The duration of collection for each set of samples was 15 minutes (15 μL volume).

2.2.3 High potassium aCSF studies

The release of Glu in the brain was achieved by high K^+ stimulation. This was accomplished by modifying the aCSF composition (20 mM of NaCl, 103 mM KCl, 20 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.25 mM Na_2HPO_4). The setup of this experiment was similar to the one mentioned above except the liquid switch was placed between the syringe pump and microdialysis probe. The liquid switch facilitated rapid switching from aCSF to K^+ spiked aCSF without any flow interruption. Samples were collected every 15 minutes.

2.2.4 Instrumentation

A Beckman Pace-MDQ system with an LIF detector at 488nm was employed for CE experiments. Fused silica capillaries (75 μm i.d., 55 cm total length, 45 cm from injection to detection window) were purchased from Polymicro Technologies (Phoenix, AZ). Before sample injection and separation, the capillary was conditioned for 5 minutes individually with 1M NaOH, water and run buffer. The sample was injected into the capillary for 4 sec by pressure injection and detected at 488nm. CE run buffers consisting of boric acid (pH 9.2) and SDS at various concentrations were evaluated to optimize the amino acid separation.

2.2.5 Sample preparation

Amino acid standards (1 mM stock) and microdialysis samples were derivatized with equal volume of 7 mM naphthalene-2,3-dicarboxyaldehyde (NDA) prepared in acetonitrile and 10 mM of NaCN prepared in 50 mM boric acid buffer (pH 9.2). The amino acid standards were individually derivatized with equal volume of NDA and NaCN, followed by mixing of equal volumes of derivatized amino acid which resulted in a mixture of the amino acid standards for analysis; the final individual amino acid concentration in the mixture was 2.6 μM. NDA and CN^- were used to derivatize the primary amine. NDA reacts with primary amines in presence of CN^- at a basic pH to produce fluorescent cyno-2-substituted-benz(f) isoidole (CBI) derivatives as shown in Figure 6. The excitation and emission maxima for CBI derivative are approximately 420/440nm and 495nm respectively [15].

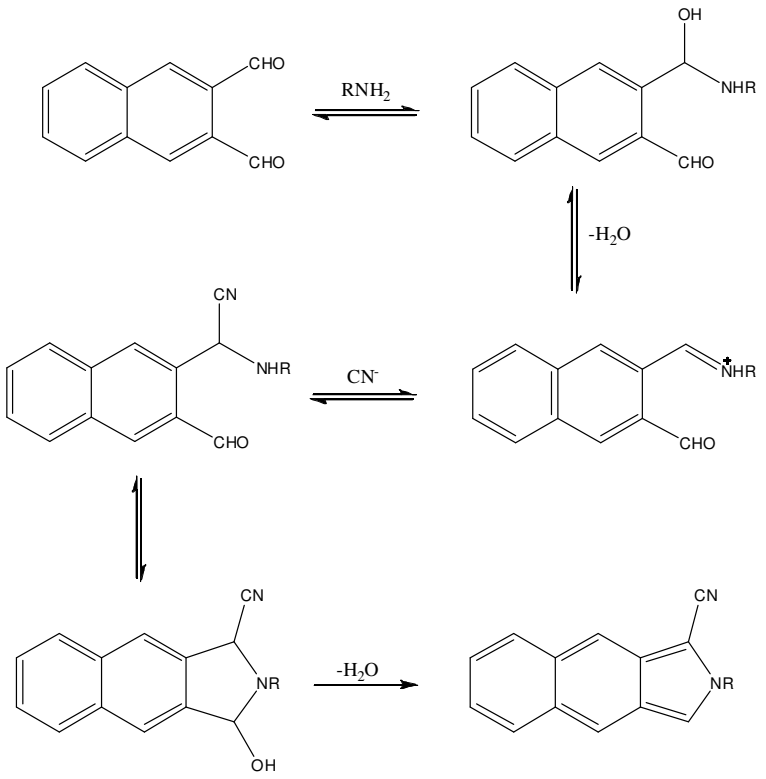

Figure 6: NDA and CN^- derivatization reaction obtained from Tom Linz with his permission [15].

2.3 Results and discussions

2.3.1 Optimization of amino acid separations

Initial, studies were performed with five amino acid standards (Gly, GABA, Tau, Glu, Asp) to evaluate the effect of SDS in the background buffer (20 mM boric acid, pH 9.2) on separation efficiency and resolution. We were unable to resolve GABA and Tau without addition of surfactant. It has been suggested in textbooks and literature that even though MEKC is the method of choice to separate neutral analytes, this technique can also significantly increase the selectivity and resolution of charged analytes [5]. Therefore, SDS was incorporated in background buffer at various concentrations to investigate its effect on resolution.

It was empirically observed that the concentration of surfactant had a significant effect on the separation as shown in Figure 7. First, the presence of SDS in the buffer increases the migration time of analytes compared to buffer with no SDS. Since the surfactant used in this experiment is anionic, the negatively charged micelle is strongly attracted to the anode (electrophoretic mobility) which is opposite to the direction of EOF. Based on this, the analytes that are less associated with the micelles will migrate earlier than the ones that exhibit stronger interaction with the micelles. The elution order of the analytes did not change after the addition of SDS (compared to no SDS buffer); this can possibly be attributed to the fact that at pH 9.2,

all the analytes were negatively charged. Also, the CBI derivative moiety imparted some hydrophobic characteristics to the analyte. Therefore, each analyte was associated at least in part with the micelle.

GABA and Tau were separated using 15 mM SDS concentration possibly due to a differences in this interaction with the SDS micelle. Also, the increase in SDS concentration helped the separation by decreasing EOF through an increase in ionic strength. All five analytes were resolved using 15 mM SDS and hence this was considered the best possible SDS concentration. The migration time of analyte vs. concentration of SDS was plotted and is shown in Figure 8. It was noted that at SDS concentrations higher than 15 mM, the migration times of Glu and Asp did not change significantly. This can be due to the fact that the partition of the CBI derivatives of Asp and Glu into the SDS micelles at concentration above 15 mM (in presence of 20 mM borate buffer) does not change.

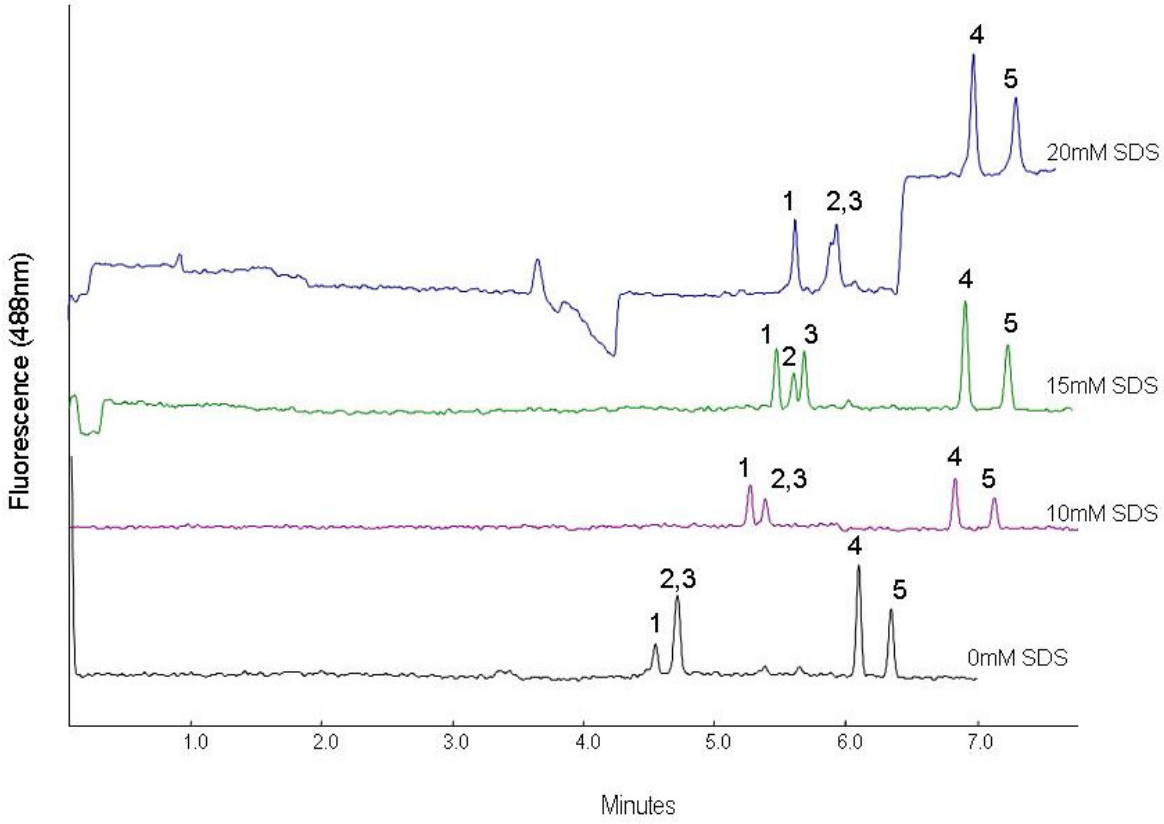

Figure 7: Optimization of SDS concentration with 20mM borate pH 9.2 for separation of amino acid standard mixture under following CE conditions: capillary length was 50 cm with 75 μm I.D.; 8 μM amino acid standard mixture of sample was injected for 4 sec at 0.7 psi and separation voltage of 20 KV. Amino acid standard mixture consisted of: 1, Gly; 2, GABA; 3, Tau; 4, Glu; 5, Asp.

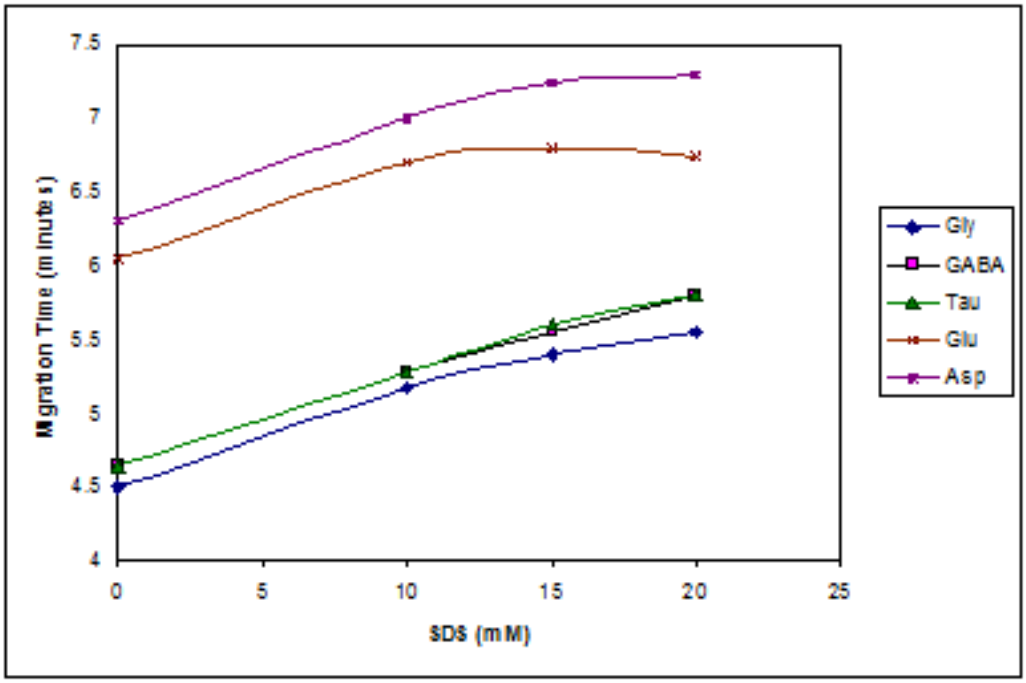

Figure 8: Comparison of varying SDS concentration for separation of amino acid standards based on migration time vs. SDS concentration plot for five amino acid standards.

Following an initial evaluation of the optimal concentration of SDS using MEKC, attempts were made to separate all fifteen amino acid standards. Overall, most of the peaks were resolved except the following pairs: (Asn and Ser), (Phe and Trp), and (Arg and Thr) as shown in Figure 9. Next, keeping the SDS concentration constant, the borate buffer concentration was systematically varied to examine the effect on the separation as shown in Figure 10. Although borate high salt concentration increases ionic strength thus enhancing buffer capacity, it decreases EOF due to a smaller ionic double layer leading to longer analysis times. However, the rationale for performing this study was to investigate the effect of buffer on separation by varying EOF and by changing the extent of interaction of the analytes with the micelles. Increased borate concentration aided in separating early eluting peaks, but not the late eluting peaks. Again, as observed in the previous study with SDS, the late eluting peaks were not affected by changes in borate concentration (with constant SDS concentration) as these analytes exhibit "strong" hydrophobic interactions with the micelle and therefore are less affected by the external environment of the micelle. After evaluation of various separation conditions, 20 mM boric acid and 15 mM SDS at pH 9.2 was deemed to produce an optimal separation. This buffer composition was used for all further experiments.

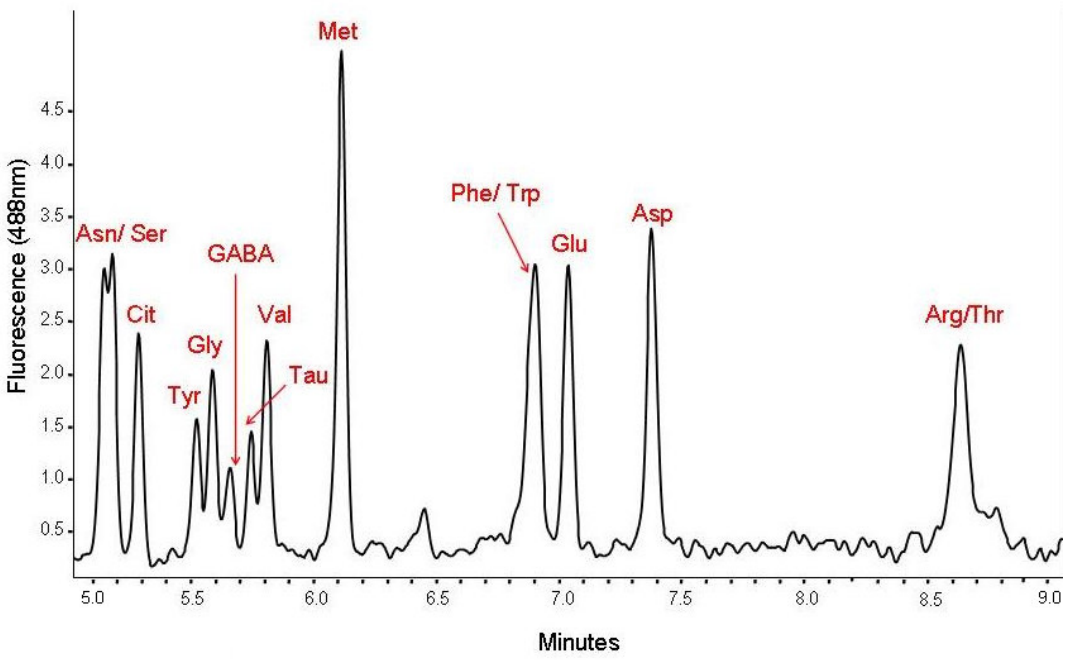

Figure 9: Electropherogram of amino acid standards mixture under optimal buffer composition (20 mM Borate 15 mM SDS at pH 9.2) with following CE conditions: capillary length was 50 cm with 75 µm I.D.; 2.6 µM amino acid standard mixture of sample was injected for 4 sec at 0.7 psi and separation voltage of 20 KV.

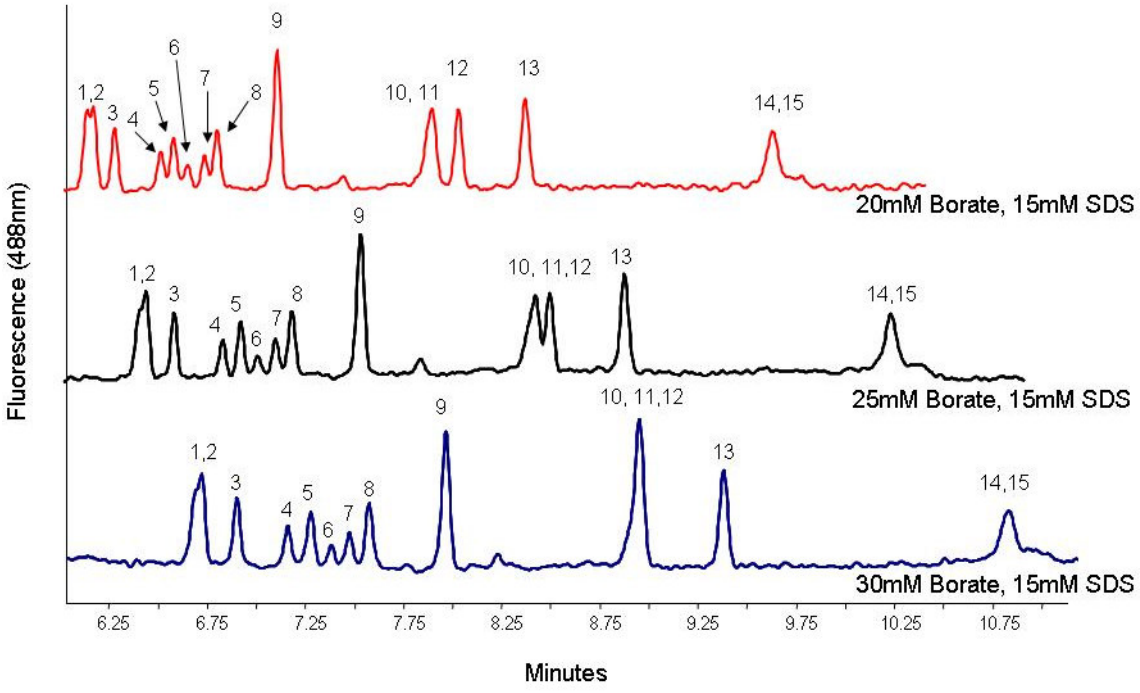

Figure 10: Optimization of buffer concentration with 15 mM SDS pH 9.2 for same separation of amino acid standard mixture under following CE conditions: capillary length was 50 cm with 75 µm I.D.; 2.6 µM amino acid standard mixture of sample was injected for 4 sec at 0.7 psi and separation voltage of 20 KV. Amino acid standard mixture consists: 1, Asn; 2, Ser; 3, Cit; 4, Tyr; 5, Gly; 6, GABA; 7, Tau; 8, Val; 9, Met; 10, Phe; 11, Trp; 12, Glu; 13, Asp; 14, Arg; 15, Thr.

2.3.2 Microdialysis sample analysis

Once the separation conditions were established with amino acid standards, basal microdialysis samples were analyzed following offline derivatization with NDA and NaCN as shown in Figure 11. For peak identification, the samples were spiked with amino acid standards (Glu, Asp, Phe) and analyzed by CE-LIF.

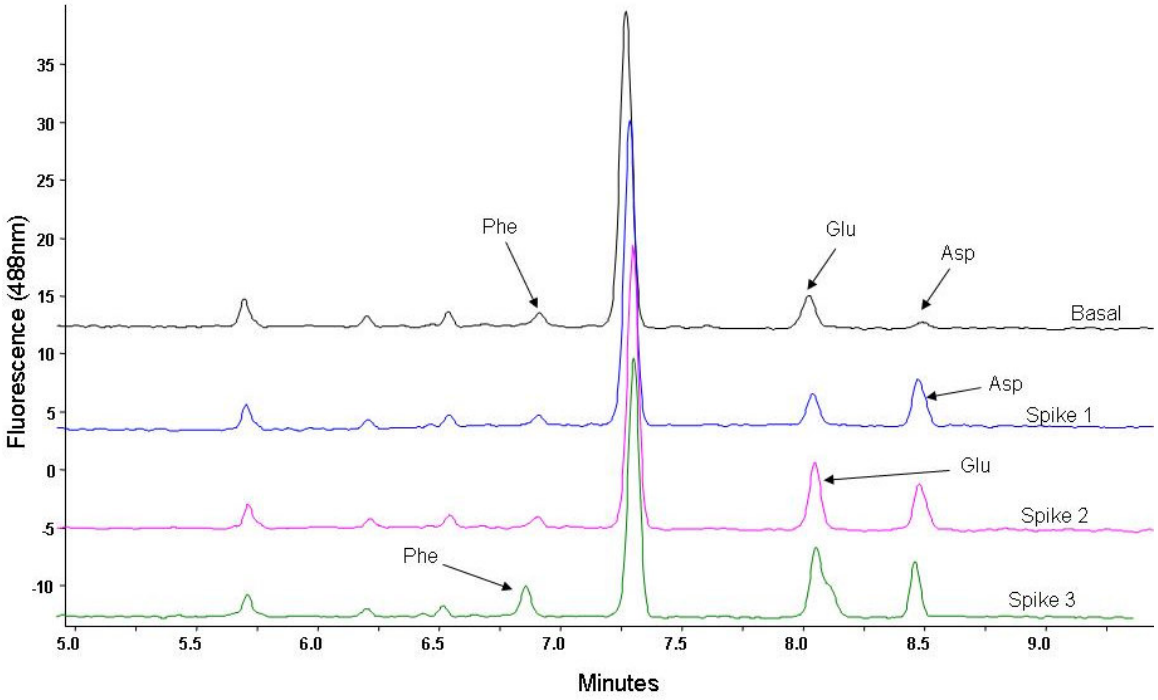

Figure 11: Electropherograms for peak identification from *in vivo* samples collected from rat brain striatum. 10 µL of microdialysis sample was derivatized with equal volumes of NDA and CN^-. The flow rate used was 1.0 µL/min. Spike 1 is the electropherogram obtained by spiking with standard Asp. In spike 2 the sample was spiked with standard Glu. In spike 3 the sample was spiked with standard Phe. CE conditions: capillary length was 50 cm with 75 µm I.D.; sample was injected for 4 sec at 0.7 psi and separation voltage was 20 KV. Buffer composition: 20 mM borate, 15 mM SDS, pH 9.2.

Additionally, microdialysate samples obtained after perfusion with K^+ spiked aCSF were also analyzed. Infusion with high K^+ aCSF in the brain has been reported in the literature to increase the concentrations of excitatory amino acids in the CNS extracellular space. Perfusion with high K^+ depolarizes the presynaptic membrane, and activates voltage-gated calcium channels allowing influx of calcium [16]. Presence of excess calcium in cell leads to the release of Glu stored in presynaptic vesicles via exocytosis process. Several groups have demonstrated an immediate increase in the concentration of brain extracellular Asp and Glu following the switch from aCSF to high K^+ aCSF. Such a method is an easy and fast stimulus to evoke a dynamic in vivo response that creates an ideal changing analyte concentration situation *in vivo* to evaluate the performance of online microdialysis CE systems. Such effects can also be produced by the administration of the Glu reuptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) that results in excess Asp and Glu concentration in extracellular space [17] .

We were interested in using this study to demonstrate a dynamic change in an endogenous neurotransmitter. Also, such studies may guide us for subsequent microchip experiments in terms of identifying the correct peak as the high K^+ can possibly change basal levels of other neurotransmitters present in the brain which may make the data interpretation complicated for online microdialysis analysis.

To observe alteration in the concentration of amino acids, the microdialysis probe was initially perfused with normal aCSF at a flow rate of 1.0 μL/min. Basal data was collected for 15 minutes following which, the perfusate was switched to high K^+ aCSF as shown in Figure 12. The electropherogram obtained for the basal sample was consistent with previous experiments with regard to migration time. An increase in the peak height of Glu was observed for the first 15 minutes sample. It has been reported in the literature that the changes in levels of neurotransmitter with high K^+ perfusion occurs within a minute. However, as these experiments were performed offline, we were not able to collect sample every minute due to volume limitations of the automated CE system. However, this experiment established proof of concept and the prospect to transfer this method to an online microdialysis-microchip setup to perform high temporal resolution analysis of fast dynamic changes of neuroactive amino acids.

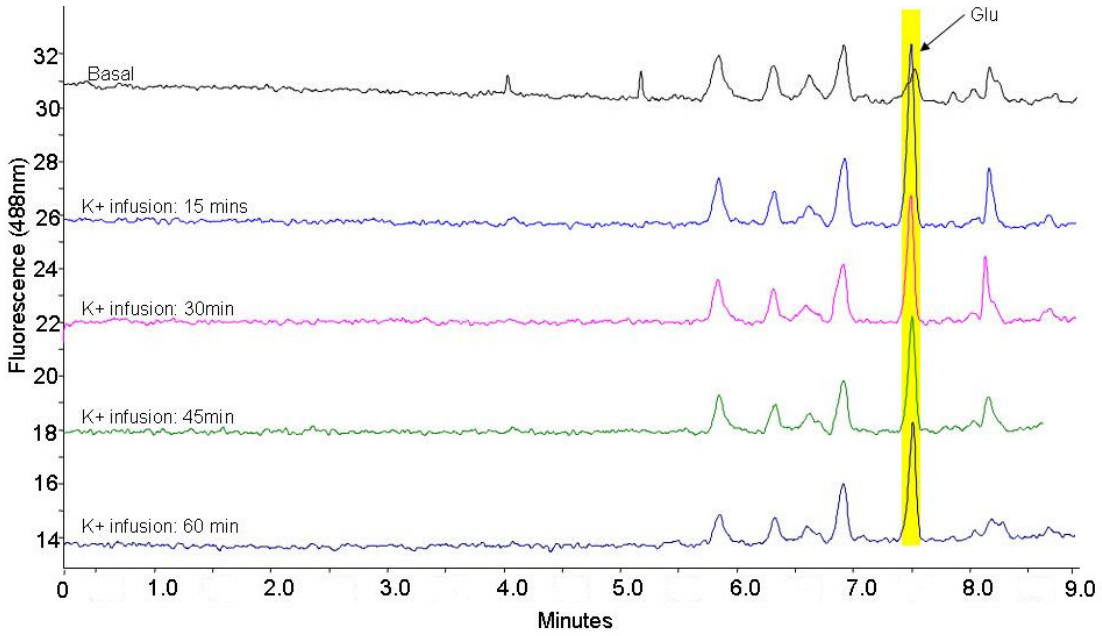

Figure 12: Monitored change in levels of Glu with perfusion of high K^+ aCSF. Microdialysis samples were collected every 15 minutes at flow rate of 1.0 µL/min and derivatized with NDA and CN. This was accomplished under the following CE conditions: capillary length was 50 cm with 75 µm I.D.; sample was injected for 4 sec at 0.7 psi and separation voltage was 20 KV. Buffer composition: 20 mM borate, 15 mM SDS, pH 9.2.

2.4 Summary

In this chapter, studies were performed to optimize buffer conditions for the separation of amino acid standards as well as Asp and Glu from rat brain microdialysis samples to provide us with some background data on the separation for microchip electrophoresis. The MEKC based method was successfully developed for the separation of amino acid standards as well as identification of Glu and Asp in microdialysis samples. These optimization studies using CE aided us in the development of a method for the analysis of microdialysis samples on the microchip electrophoresis setup by being able to explore similar buffer conditions. Also, we were able to monitor *in vivo* changes in Glu concentration by perfusing the rat brain with high K^+ aCSF signifying the feasibility of studying an *in vivo* dynamic change with online microdialysis-microchip setup.

2.5 References

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CHAPTER 3

MICROCHIP ELECTROPHORESIS

3.1 Introduction of microchip electrophoresis

Microchip electrophoresis (MCE) is a mode of CE in which analysis is performed in a miniaturized device containing microchannels. The concept of MCE was first introduced by Harrison et, al. [1] ; since then the microchip format has been used as an analytical platform for a wide spectrum of applications including small molecule separations [2,3], protein [4-7] and peptide [8] analysis, DNA analysis [9,10], and immunoassay [11]. Although in the true sense, MCE can be considered as a simple scaled down version of CE, the true potential of these devices lie in their ability to perform analysis of volume-limited samples. The quantity of sample actually injected into the microchannel of a chip device is typically in the picoliter (pL) range compared to nanoliter (nL) in CE and microliter (μL) range in LC. Also for most applications, the field strength is higher due to short separation channels (few cm), therefore high resolution separations can be performed within a few seconds to minutes. In addition, the small channel and reservoir dimensions result in reduced reagent consumption for on-chip reactions. For incorporation of on-chip sample manipulation (i.e derivatization, preconcentration, cleanup), additional channels can easily be added into a design already containing electrophoresis channels.

Various detection schemes have been used in microchip devices, the most popular and sensitive among these is fluorescence detection [12]; such arrangements

generally involve a laser of a particular wavelength as an excitation source that is aligned and focused through suitable optics onto a point within the separation channel for the detection of fluorescent analyte plugs. Other examples of detection systems involve electrochemical detection, chromatography, and MS which is beyond the scope of the present study [12].

The objective of the work in this chapter was to develop an easily fabricated microchip device that can be coupled to microdialysis sampling. Online derivatization was incorporated to this system prior to the electrophoresis as the continuous sampling from the brain of an anesthetized rat were performed. Several factors need to be considered during the development of such a design. These includes (i) building an interface that will be able to deliver hydrodynamic flow (1 μL/min per flow from a syringe pump) into the chip microchannels for further manipulation, (ii) derivatization of the sample by suitable reagents to generate fluorescent derivatives, (iii) incorporation of appropriate sample injection method for analysis, and (iv) separation and detection of the analytes of interest.

In the first online microdialysis microchip setup described in the literature [13], a commercially available microtight union was used to couple hydrodynamic flow to the chip. This chip was fabricated by thermal bonding of two pieces of sodalime glass. Discrete plugs of sample were introduced into a 3 cm separation channel using gated injection from the continuous microdialysis stream. The system

was used to monitor the activity of the enzyme β-galactosidase. This enzyme catalyzes the hydrolysis of fluorescein mono-β-galactoside (FMG) to fluorescein. The lag time was defined as a time needed for the device to respond to a concentration change on this device was 5–7 minutes. A similar setup consisting of a hybrid PDMS-glass device was used for online sampling, in-channel derivatization, and detection of amino acids and peptides [14]. On-chip labelling of analytes was accomplished using NDA and 2-mercaptoethanol (2ME) and injection was achieved using gated injection. Using this device a temporal resolution of 30–40 sec was obtained.

The only report of *in vivo* monitoring using a microdialysis-microchip electrophoresis system was performed by Kennedy's group [15]. The chip device was fabricated out of borosilicate glass and reaction channels were incorporated into the design for precolumn derivatization with OPA and β-mercaptoethanol of the Glu from microdialysis sample. This chip also used Upchurch fittings for fluid interfacing.

The use of PDMS based soft pneumatic valves to continuously inject sample from a microdialysis stream was demonstrated by Li et al [16]. In this report the continuous injection and separation of fluorescein and dichlorofluorescein was accomplished with temporal resolution of 20 sec. All fluids were delivered from the syringe pump or from the probe using a fused silica capillary. The approach was also

utilized to monitor the release of dopamine from PC12 cells after stimulation using amperometric detection [17].

The generation of aqueous plugs contained in a continuous flow stream of oil for microdialysis sampling has been evaluated by Kennedy's group [18]. This approach was employed to create reaction chambers within the droplets, and to minimize loss of temporal resolution due to Taylor dispersion within the connecting tubing. With this system, glucose was measured online *in vivo* from rat brain using glucose oxidase and Amplex Red. A similar approach incorporating a K-shaped injection interface was evaluated for continuous injection and separation of amino acid standards from droplets generated via segmented flow. NDA/CN was utilized to derivatize the primary amines [19].

In this chapter, an online microdialysis-microchip system was developed in a systematic manner. First, the parameters in separation channels were characterized with respect to fabrication reproducibility and analysis performance. Second, the device was interfaced with hydrodynamic flow. Following that, a derivatization scheme was incorporated and optimized for NDA, CN^-, and microdialysis sample/amino acid standards. Finally the chip was utilized to carry out online analysis both *in vitro* and *in vivo*.

3.2 Experimental

3.2.1 Microchip fabrication

The fabrication of PDMS microchips involves several steps as shown in Figure 13. First, the microchip designs were drawn using AutoCad software. Second, high resolution (50,000 dpi) photomasks with the required designs (in negative tone) were obtained from IGI (Minneapolis, MN). Using this mask, a master or a mold was prepared on a silicon wafer. The process starts out by aligning a 4inch silicon wafer on the chuck of a spin coater. The approximately 4 mL of SU8-10 (negative photoresist purchased from Microchem) was placed on silicon substrate with the aid of a dispensing syringe. The following coating cycle was followed (500 rpm for 5 sec followed by 1300 rpm for 30 sec). Then, the wafer was soft baked on a hot plate at 65^{o}C for 3 minutes and 95^{o}C for 15 minutes. After waiting for 5 minutes for the wafer to cool to room temperature, it was aligned with the negative photomask containing the chip design using a mask aligner, and was exposed to UV light for 17 sec. The exposed wafer was then transferred to a hotplate for postbake at 95^{o}C for 15 minutes. The wafer was allowed to come to room temperature following which it was developed in 10 mL of SU8 developer (Microchem, Newton, MA) for approximately 5 minutes. At this point the raised features are clearly visible on the silicon wafer. The wafer was rinsed thoroughly with copious amounts of IPA followed by nanopure water. At this point, if cloudiness was observed on the surface

surrounding the features, the wafer was developed for an additional 2 minutes followed by water washes until the cloudiness disappeared. Finally, the wafer was hard baked on a hot plate at 250^{o}C for 2-3 hrs for polymerization and hardening of the photoresist features. This wafer mold was utilized for the fabrication of PDMS chips.

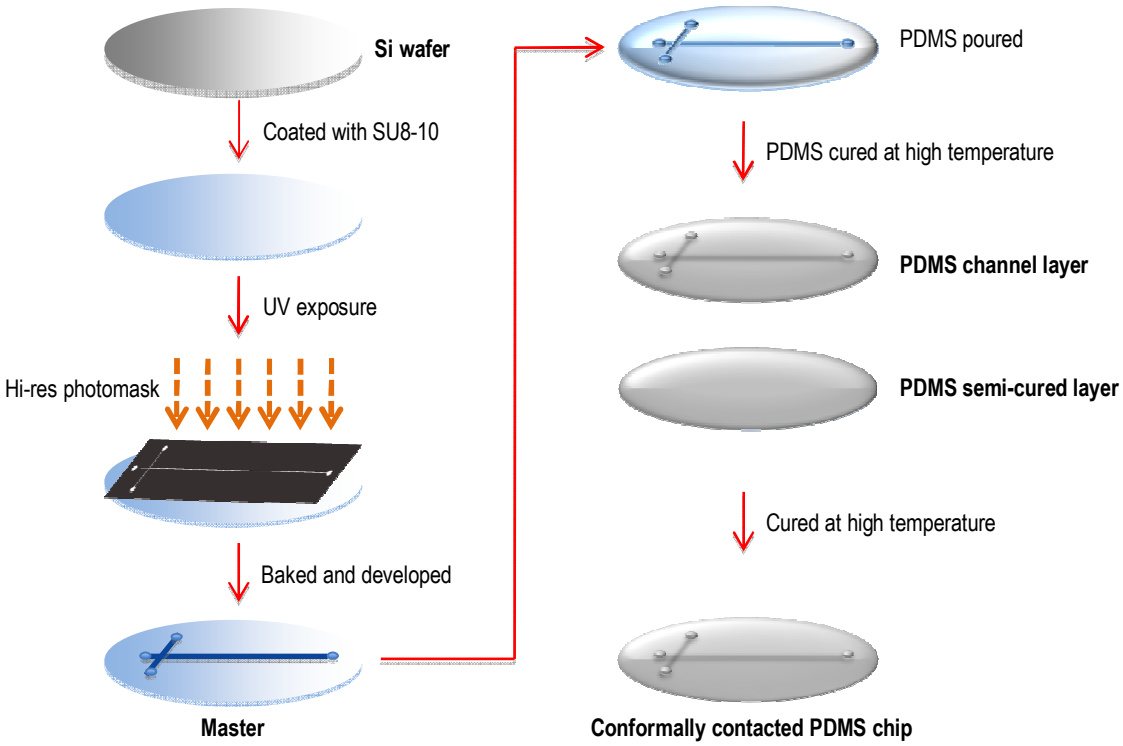

Figure 13: Fabrication procedure for PDMS microchip obtained from Pradyot Nandi.

3.2.2 Microchip design and interface

The chip devices used in this study were constructed out of two separate layers of PDMS. An approximately 5 mm thick layer of a mixture of PDMS and curing agent (10.5 g:1.5 g) was poured on the master containing the channel for the channel layer followed by curing for 25-30 minutes at 90^{o}C in a convection oven. The cured channel layer was peeled from the silicon master and reservoirs were punched with biopsy punches (4 mm in diameter). Inlets for hydrodynamic flow were punched with 20G luer stub adapter (BD, Franklin Lakes, NJ). Another thin (approximately 1mm) blank layer was semi-cured in the oven at 95^{o}C for 15-18 minutes until the layer is tacky (sticky but does not deform with touch). Then both layers were contacted carefully; if air pockets were observed between the layers, the wafer was tapped very gently on the counter without applying any physical pressure. Once uniform conformal contact was seen, the chip was placed in the oven at 85^{o}C overnight for semi-permanent contact.

Channels were incorporated into this device for NDA and CN^- for derivatization. The channels were interfaced with hydrodynamic flow inlets using steel pins, which were placed in each inlet for NDA, microdialysis and CN^- flow as shown in Figure 14. The opposite ends of pins for NDA and CN^- flow were connected to the 1mL syringe (housed in a syringe pump, CMA 400) via FEP tubing. The microdialysis flow was connected to outlet of a microdialysis probe via FEP

tubing. The individual flow rate for each of the 3 components was optimized at 1 μL/min per flow (total flow of 3 μL/min) for delivery to the chip device. The optimized width of the derivatization serpentine channel was empirically determined as 120 μm in order to avoid pressure buildup that could result in delamination of contacted PDMS layers. A 2 mm diameter reservoir was created at the junction of the derivatization and injection channel; this served as the sample pool for derivatized sample. Such a design made device optimization easier as the derivatization flow (hydrodynamic) and separation step were independent of each other. Also, the simple injection reported by Zhang et. al. [20] was employed on this design without any complicated and time consuming optimization studies associated with gated injection.

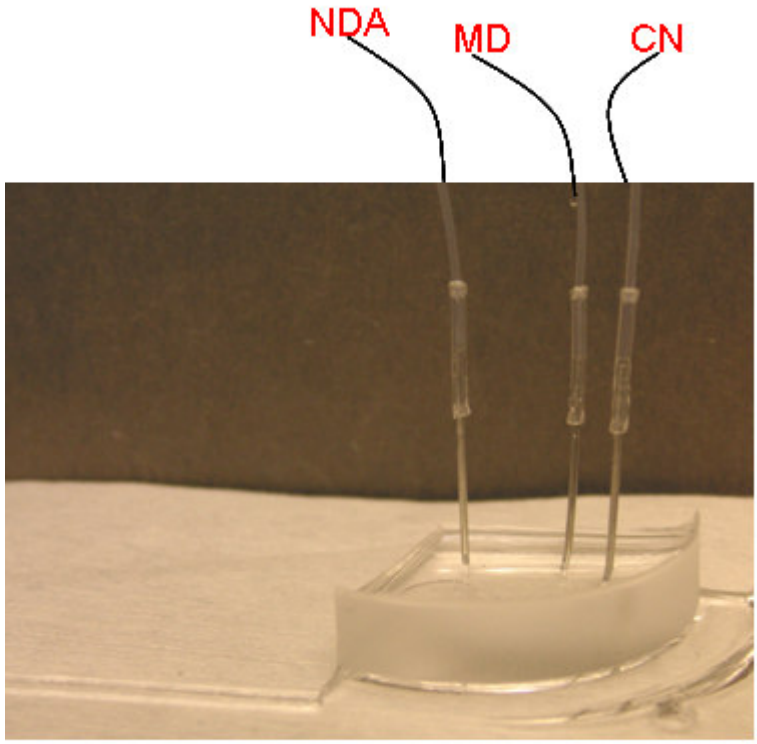

Figure 14: Interface of hyrodynamic flow for NDA, CN^- and microdialysis sample on micofluidic device; the steel pins are connected to syringe pump via FEP tubings.

3.2.3 Sample injection

For offline experiments, an injection voltage of 50 V/cm for 0.5-1 sec was applied across the channel from left to right, and separation voltage of 400 V/cm was applied across the serpentine separation channel. This same voltage scheme was followed for online experiments except that the sample from sample pool reservoir was manually discarded and allowed to fill up approximately for 30 sec before each injection.

3.3 Offline results

3.3.1 Optimization separation of amino acid standards

Fabrication of chip devices has been investigated using various materials. The most popular among them is the use of a rubber-like optically transparent polymer called (poly)dimethylsiloxane (PDMS). The other substrate that is frequently used is glass (sodalime or borosilicate), which is an ideal substrate for EOF generation that is essential for CE separation. Also, glass devices are more robust and have longer lifetimes compared to PDMS. However, fabrication of glass microchips can be quite challenging due to a high-temperature thermal bonding step which is necessary for bonding of two plates of glass. This step is not very reproducible and results in a high failure rate. On the other hand, PDMS is inexpensive and chip devices can be

fabricated quite easily based on standard photolithographic techniques. The disadvantage of using PDMS is the gradual deterioration of EOF over prolonged usage due to the surface modification and analyte adsorption [21].

In previous reports, our group employed a glass microchip with 3 cm separation channel for *in vitro* analysis of amino acid/peptide standard solutions [14]. In order to completely resolve the amino acids of interest (Glu and Asp) from other primary amines present in the complex brain microdialysate, a 20 cm long serpentine channel with 15 μm wide sampling channel in PDMS was fabricated and characterized for offline analysis of amino acid standards and a microdialysis sample as shown in Figure 15. The narrow sampling channel prevented the leakage of sample into the separation channel during analysis. Both standards and microdialysate were derivatized with equal volumes of 7 mM of NDA dissolved in acetonitrile and 10 mM of NaCN dissolved in 50 mM of boric buffer at pH 9.2.

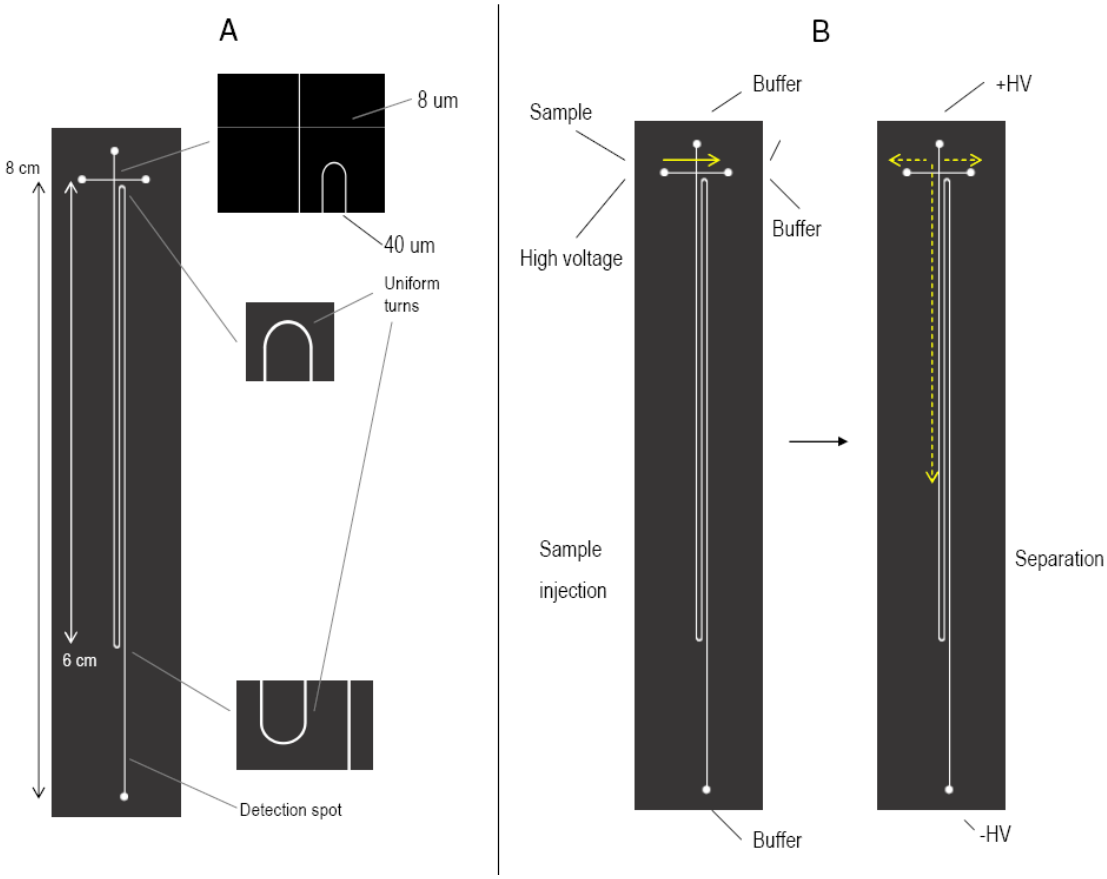

Figure 15: A) Serpentine microchip electrophoresis design for offline separation of amino acid standards and microdialysis sample. B) A yellow arrow shows the sample injection scheme from left to right, and a dashed yellow arrow indicates the separation scheme from top to bottom.

It has been reported in literature that addition of SDS in the run buffer reduces adsorption of analyte to PDMS and generates faster EOF [22]. Since our interest was to separate neuroactive amino acids (specifically Glu and Asp) in brain microdialysate, the buffer composition of 200 mM boric acids, 80 mM SDS and 5% acetonitrile was experimentally considered optimal as the background buffer. Although high ionic strength buffers are not commonly used for CE separations, as application of high voltages using such buffers leads to high currents with subsequent Joule heating, in our experiments we did not encounter any problems associated with heating such as creation of bubbles. Also, the use of such high salt buffers has been reported for microchip CE [23]. This can be explained in terms of gas permeability properties of PDMS (compared to glass) which provided efficient heat exchange with the surroundings.

Another important driving factor regarding the incorporation of SDS was that the filling of the serpentine hydrophobic PDMS channels was more easily accomplished with this buffer and did not require application of a vacuum. Under these buffer conditions Glu and Asp came out around 1.5 minutes in the middle of other amino acids as shown in Figure 16. Therefore, further optimization of the separation was done with different buffer condition. Using a buffer composition of 20 mM boric acid and 15 mM SDS, Glu and Asp eluted last. In addition, their migration time was faster compared with the other buffer composition as shown in Figure 17. However, it was noted that for the channel to fill up with this buffer was

substantially more challenging and required controlled and intermittent application of vacuum for uniform fill.

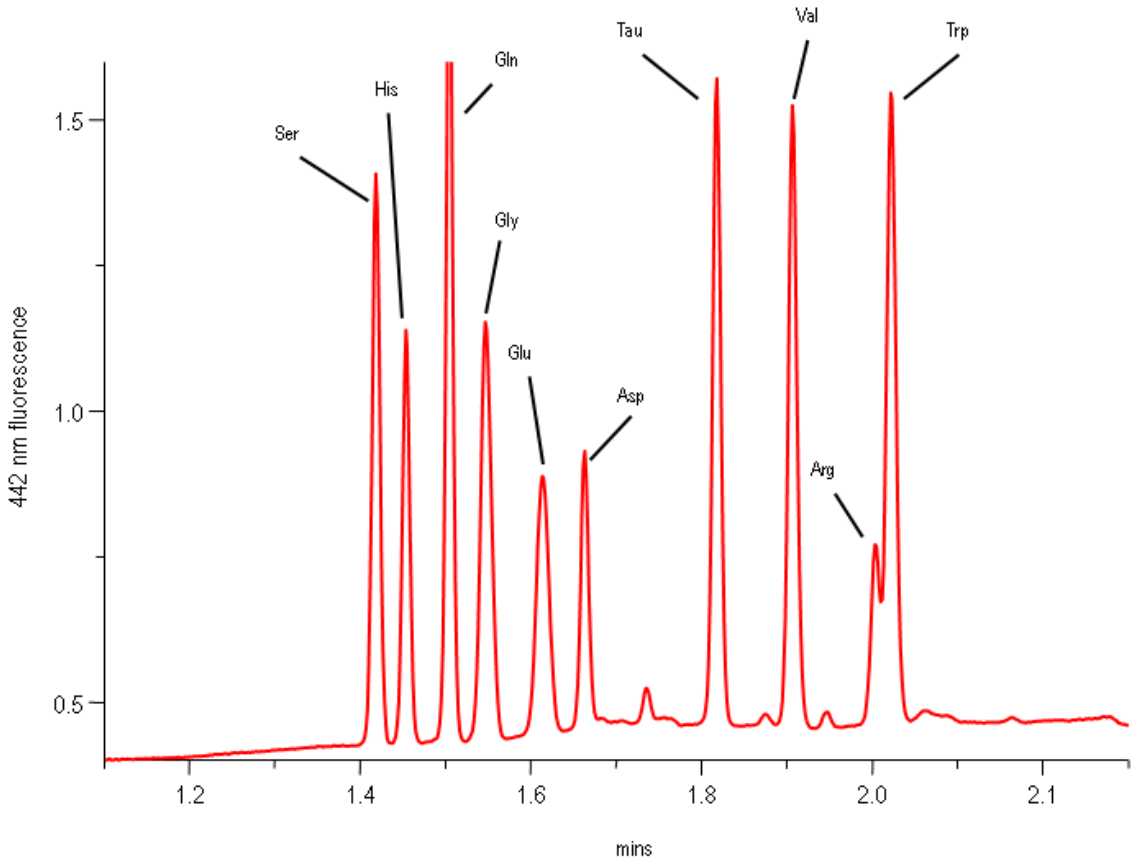

Figure 16: Electropherogram of offline separation of 1 µM amino acid standards derivatized with NDA and CN^- under following separation conditions: 200 mM boric acid; 80 mM SDS; 5% acetonitrile; pH 9.2; 400 V/cm.

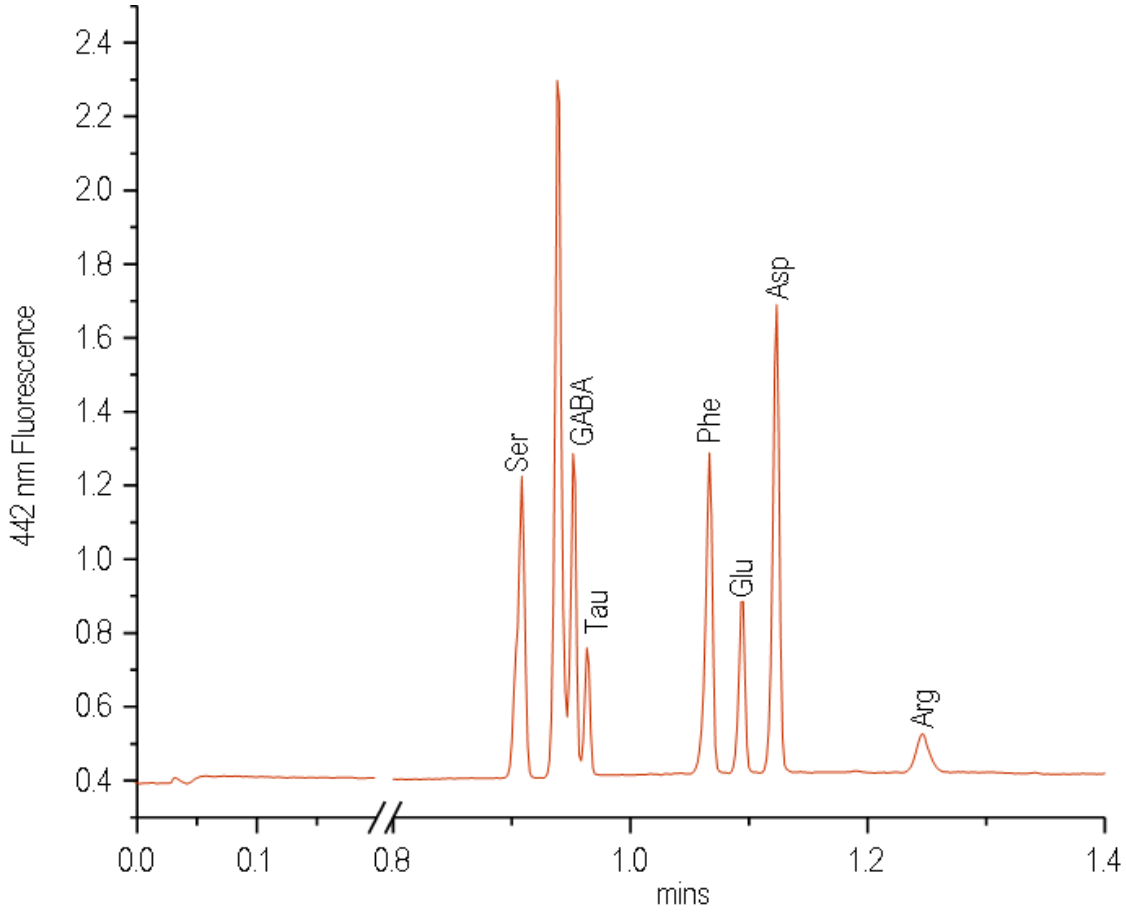

Figure 17: Electropherogram of offline separation of 2.1 µM amino acid (except Asp 4.2 µM) standards derivatized with NDA and CN^- under following separation conditions: 20 mM boric acid; 10 mM SDS; pH 9.2; 400 V/cm.

3.3.2 Detection of amino acids from microdialysis samples

Once the separation conditions were optimized for Glu and Asp, microdialysis samples were collected from a rat brain striatum, derivatized offline with NDA and CN^- and subsequently analyzed on the chip. The microdialysis sample was spiked with amino acid standards for peak identification as shown in Figure 18.

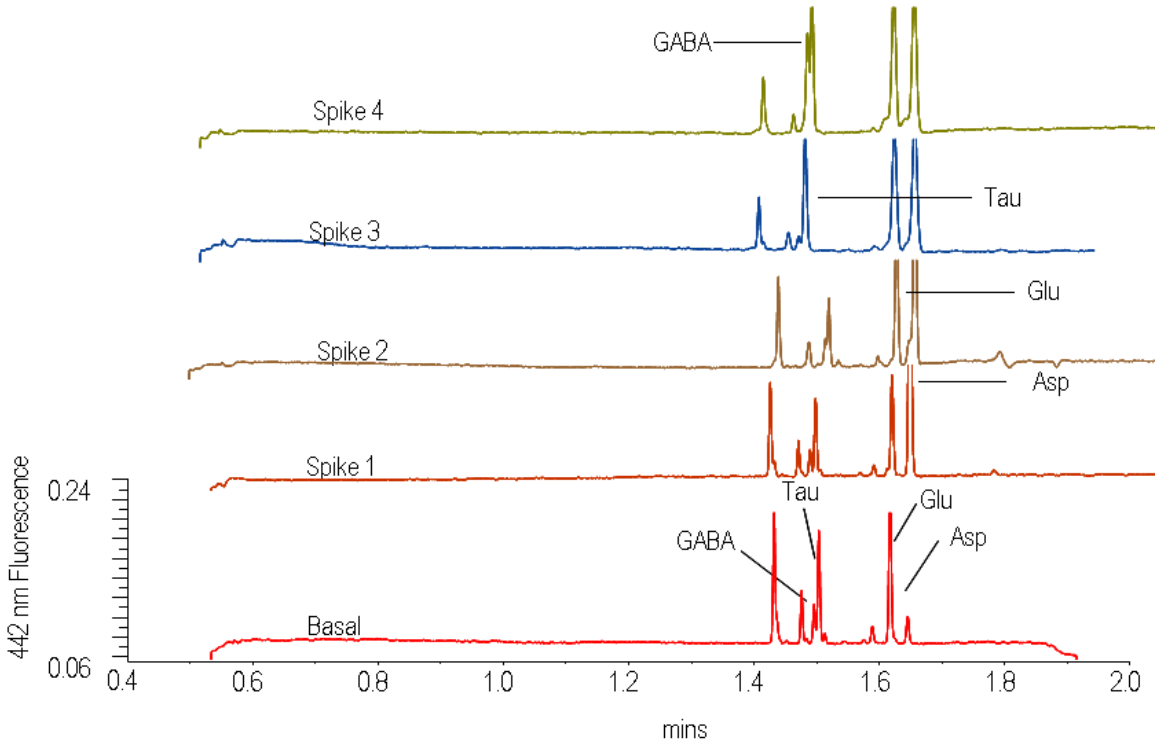

Figure 18: Peak identification in microdialysis sample collected and derivatized with NDA and CN^- offline under following separation conditions: 20 mM boric acid; 15 mM SDS; pH 9.2; 500 V/cm. Basal microdialysis sample was spiked with individual prederivatized amino acid standards (Asp, Glu, Tau, GABA).

3.3.3 K^+ spiking

Changing the perfusate from normal aCSF to high K^+ aCSF causes the release of excitatory amino acids and is therefore a good test system for the analysis system. The setup of this experiment was similar to the other offline experiment except a liquid switch was placed between the syringe pump and microdialysis probe. Microdialysis samples were collected every 15 minutes, and derivatized offline with 7 mM of NDA and 10 mM of CN^-. They were then injected and analyzed on chip. In order to identify the changes in concentrations of amino acids the microdialysis probe was first perfused with aCSF and then switched to high K^+ aCSF. This data was compared to previous data obtained from the spiked basal microdialysis sample for comparison as shown in Figure 19. An increase in Glu concentration was detected within the first 15 minutes. It has been reported in literature that changes of neurotransmitter levels with high K^+ perfusion occurs less than a minute. However, we did not see the change spontaneously because of the length of time (15 minutes) over which sample was collected; the rapid change would be detected if the sample was collected every minute. This experiment demonstrated the feasibility of performing in vivo experiments involving dynamic change on microdialysis-microchip chip device.

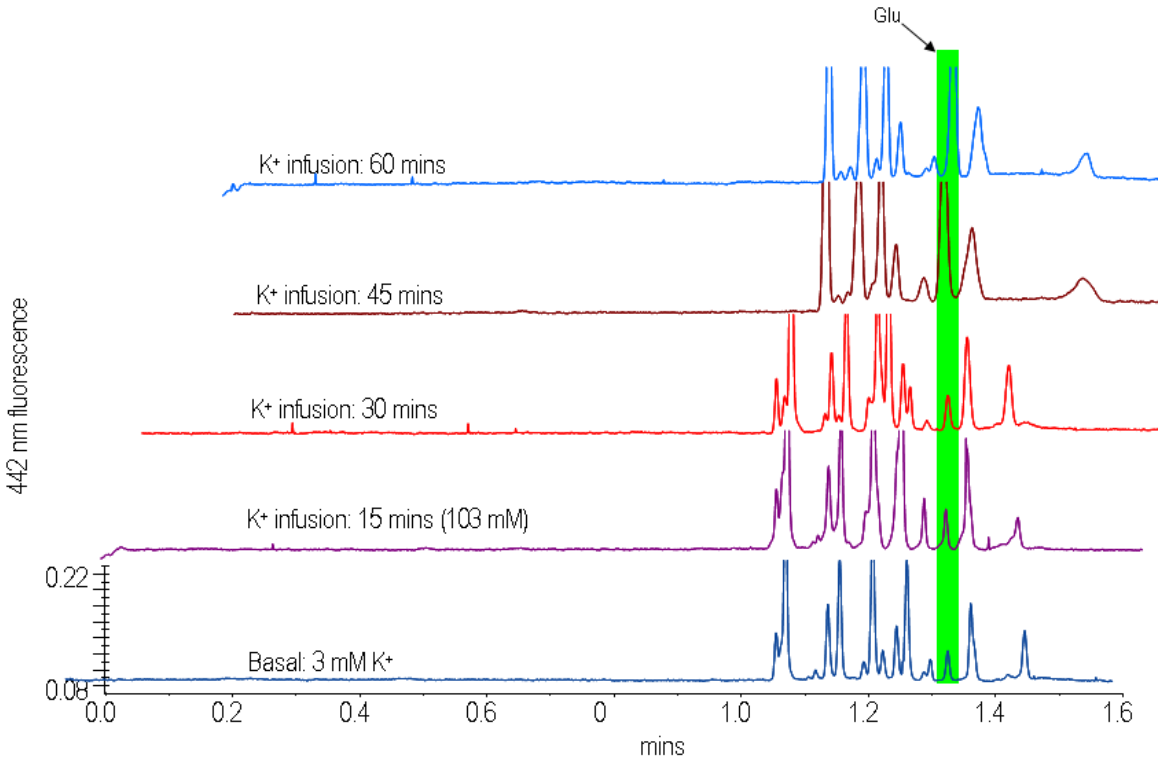

Figure 19: Offline microdialysis sample collected every fifteen minutes with infusion of microdialysis probe with high K^+ aCSF. Separation condition: 20 mM boric acid; 15 mM SDS; pH 9.2; 500 V/cm.

3.4 Online results

3.4.1 Online set up

Online analysis offers many advantages over offline analysis; this includes sample handling, introduction of sample, continuous analysis and increase in temporal resolution [24]. The online *in vivo* monitoring setup was adopted from previous work in Lunte group based on *in vitro* setup. It consists of three main components: perfusion device, sampling system and microfluidic analysis system as shown in Figure 20. The first component was the perfusion device, a syringe pump was used to continuously perfuse the microdialysis probe with aCSF. The second component the microdialysis probe that was implanted in the rat brain striatum. Lastly, sample was delivered and derivatized online with NDA and CN to microfluidic device as shown in Figure 21, which contains the derivatization channel, followed by simple cross injection, separation, and detection with fluorescence detection.

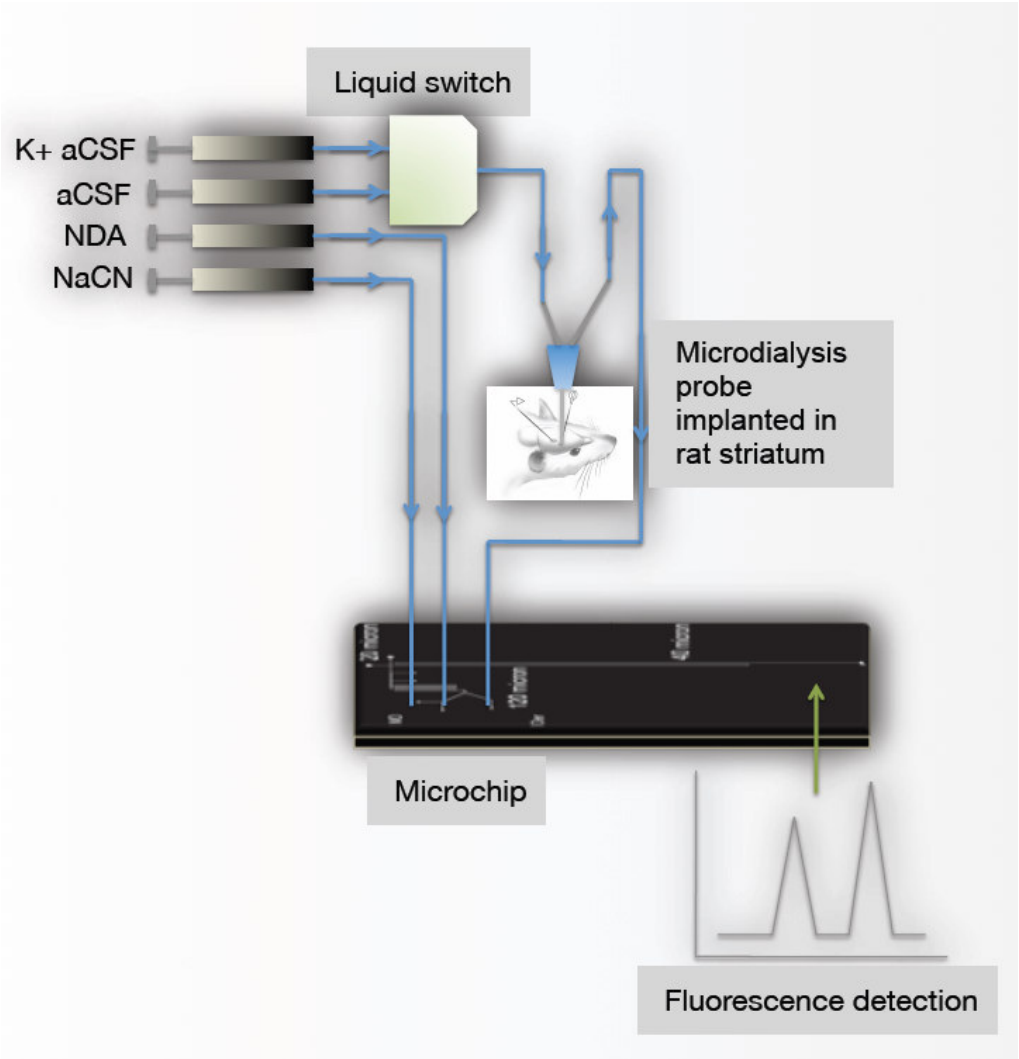

Figure 20: Basic setup for online *in vivo* monitoring obtained from Pradyot Nandi. It consists of three main components: perfusion device, implantation of microdialysis probe, and analysis of amino acids with fluorescence detection.

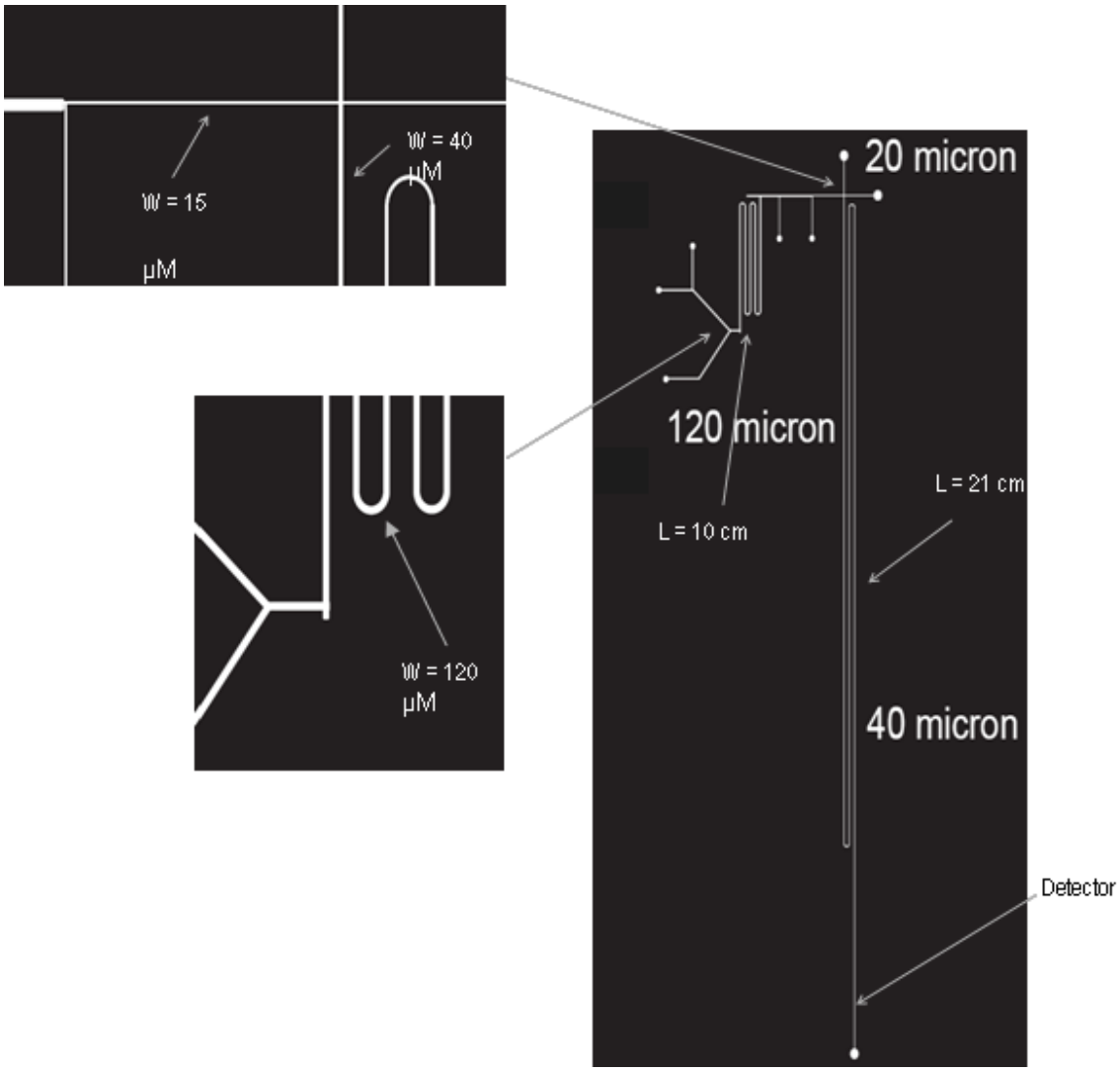

Figure 21: Microdialysis-microchip electrophoresis design for online monitoring. Left portion of the design is designated for the derivatization reaction. The derivatized sample is being collected in the reservoir and sample is injected across the channel and separated.

3.4.2 Evaluation of Mixing

One of the important challenges associated with online dervatization in the microchip is to adequately mix the three flows. To demonstrate the efficient and fast mixing with easy fabrication compared to previous techniques reported in literature [25], three different color dyes were introduced in each inlet for the reaction channels representing CN^-, microdialysis, and NDA flows. Micrographs were taken at various positions within the reaction channels as shown in Figure 22. At the Y intersection, three separate streams are observed each at 1 μL/min. The micrograph taken at upper turns illustrates that within the first turn there is no homogenous color (the three dyes are not properly mixed). However, within the second channel approaching the second turn the color becomes homogenous. The same phenomena was observed in the micrograph taken at bottom turns. The fact that there is not homogenous mixing in the microfluidic device at the beginning of derivatization channel is due to the low Reynolds number of the hydrodynamic flow. Incorporation of frequent turns generated turbulent flow. Overall, this experiment proved that proper mixing of three fluids occurred in the reaction channels within 2 minutes.

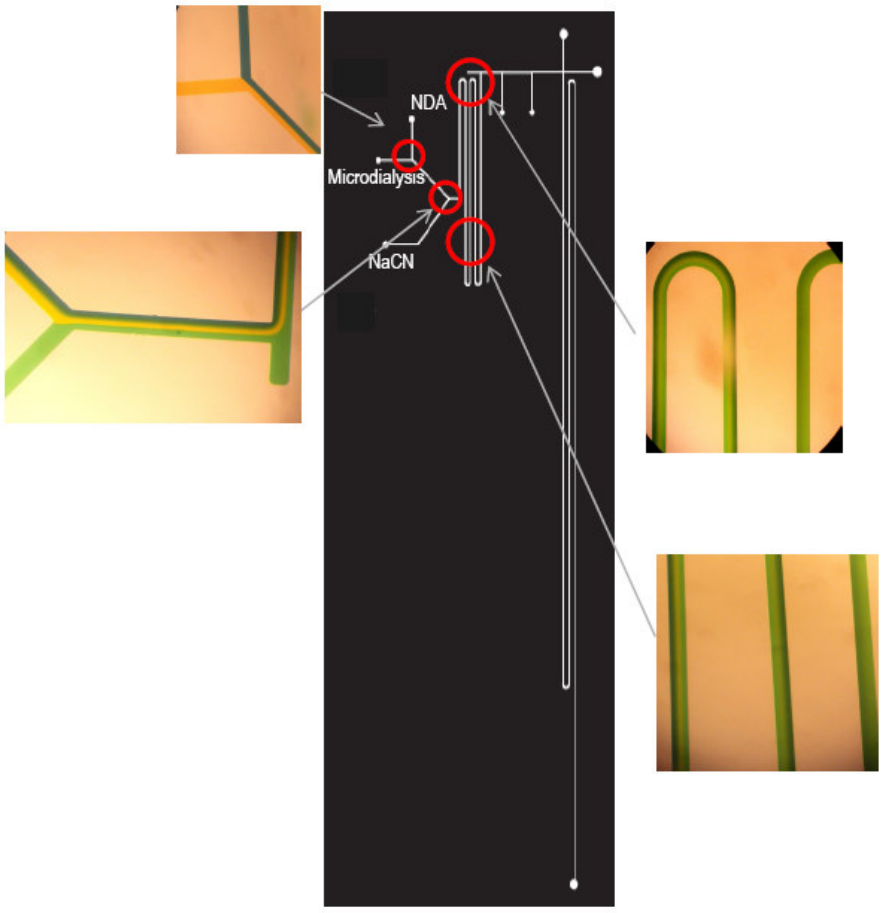

Figure 22: Demonstration of mixing with three different dyes representing NDA, microdialysis sample, CN^- flows within reaction channels. The square pictures are the micrographs taken at various points within the derivatization channels.

3.4.3 Online sampling and derivatization with concentration change *in vitro*

There are a few reports in literature using online microdialysis-microchip electrophoresis to detect a change in concentration. Huynh et al. demonstrated an *in vitro* change in the concentration of fluorescein from 50 μM to 100 μM with lag time of approximately 6.5 minutes [13]. Kennedy et al. took step further by illustrating step change in Glu levels from 0 to 100 μM with various flow rates [15]. To the best of my knowledge no one has yet demonstrated the online derivatization and separation of multiple amino acids with step change in concentration. To determine if the microdialysis microchip electrophoresis is capable of detecting concentration changes; online sampling and derivatization was evaluated *in vitro* for a change in concentration of six amino acids (Glu, Asp, Gly, Tau, Arg, Trp).

The microdialysis probe (4mm length) was placed a vial containing the amino acids of interest. The inlet of the probe was connected via FEP tubing to a 1 mL syringe containing water, and the output of the microdialysis probe was connected to the microfluidic device. The initial concentration of the amino acids was 500 nM, and was increased to 5 μM. Continuous injections were made to monitor the step change with temporal resolution of approximately 7 minutes and separation time of approximately 3 minutes as shown in Figure 23. The lag time was approximately 25 minutes for the system to detect the change. There are few factors that contributed to the longer lag time compared to previous reports, including dead volume from longer

tubing, slower flow rate and manual switching of electrodes. This demonstrates that system is capable for detecting the changes.

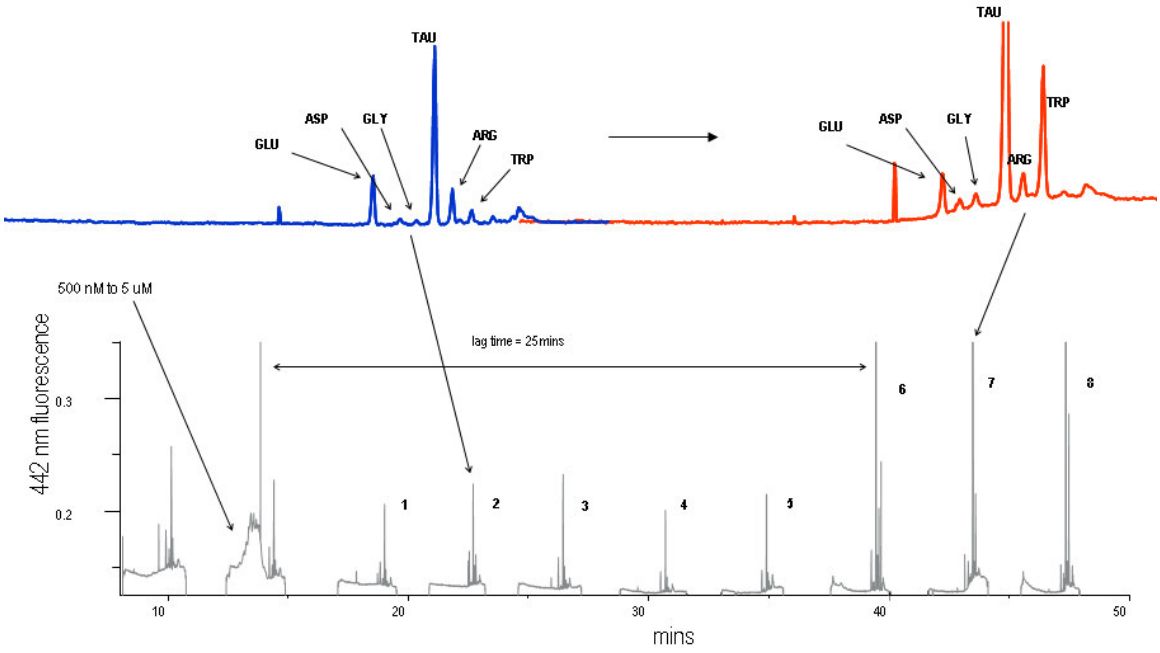

Figure 23: Concentration change *in vitro* with six amino acid standards (Glu, Asp, Gly, Tau, Arg, Trp) dissolved in water with temporal resolution of approximately 7 minutes, separation time of approximately 3 minutes and lag time of 25 minutes. Separation conditions: 200 mM boric acid; 80 mM SDS; 5% acetonitrile; pH 9.2; 500 V/cm. Initial concentration was 500 nM and later increase to 5 µM.

3.4.4 *In vivo* monitoring of amino acids

The next goal was to demonstrate the potential of microdialysis coupled to microchip electrophoresis for *in vivo* monitoring of Glu, Asp and fluorescein. This is the first time an electrophoregram of continuous injections of microdialysis samples on a same chip has been reported. Fluorescein has commonly been used as a marker for permeability of the BBB. In this experiment fluorescein was monitored along with amino acids. Fluorescein crosses the BBB by passive transport. During stroke, the tight junctions that make up the BBB are compromised due to glutamate excitotoxicity. Therefore, under stroke conditions, more fluorescein will cross the BBB.

In these experiments, the rat was injected with a bolus does of 0.5 mg fluorescein via femoral vein. The amount of fluorescein in the brain was monitored with online system. Fluorescein was detected in the microdialysate within 5 minutes after the injection implying that it crossed the brain barrier quickly, as shown in Figure 24. The fluorescein response was monitored for over 90 minutes and plotted as a function of time. It can be seen that there is a decrease in fluorescein concentration in the brain over time suggesting clearance from the brain. It has been reported in a literature that fluorescein is eliminated from the body mainly through renal clearance [26,27].

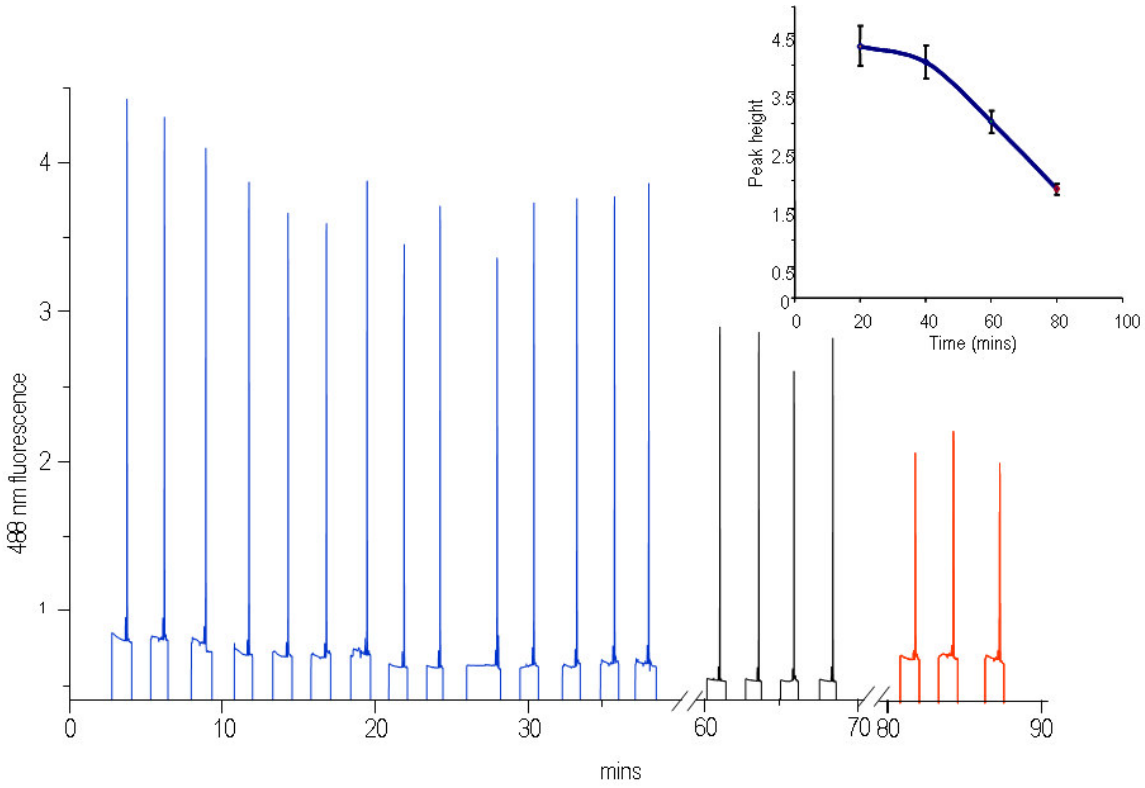

Figure 24: Continuous online monitoring of fluorescein over time with temporal resolution of 3 minutes. Separation conditions: 200 mM boric acid; 80 mM SDS; 5% acetonitrile; pH 9.2; 400 V/cm. A rat was injected with bolus dose of 0.5 mg fluorescein through the femoral vein. Peak height vs. time plot in upper right corner illustrates clearance of fluorescein over time.

At this point the preliminary experiments were concluded. Therefore, we decided to take the device a step further towards our ultimate goal of continuous online *in vivo* sampling and derivatization of amino acids followed by analysis on a microfluidic chip device. For *in vivo* microdialysis sampling, a microdialysis probe (4mm) was implanted in a rat striatum and perfused with aCSF at a flow rate 1.0 µL/min. The inlet of the probe was connected to a 1 mL syringe containing aCSF via FEP tubing, and the output of the probe was connected to microfluidic device. Fluorescein was injected into the rat based on the method mentioned above. A representative electropherogram from this study is shown in Figure 25.

As can be seen by Figure 25, we were able to obtain reproducible runs after an initial equilibration period as is demonstrated by first 2 sets of injections. These data are in accordance with the offline analysis studies where peaks were identified based on migration time and spiking with standard compounds. We were able to run these chips continuously for approximately 2 hrs. During this time there was no evidence of loss of resolution over time as a result of ability to replenish the buffer in the reservoirs on this chip. In comparison, this is a concern associated with gated injections regarding not being able to replenish the buffer frequently as sample injection is separation is performed continuously for high temporal resolution. Microdialysis was successfully coupled to microfluidic device for monitoring of Asp, Glu and fluorescein from microdialysate samples obtained from the rat striatum.

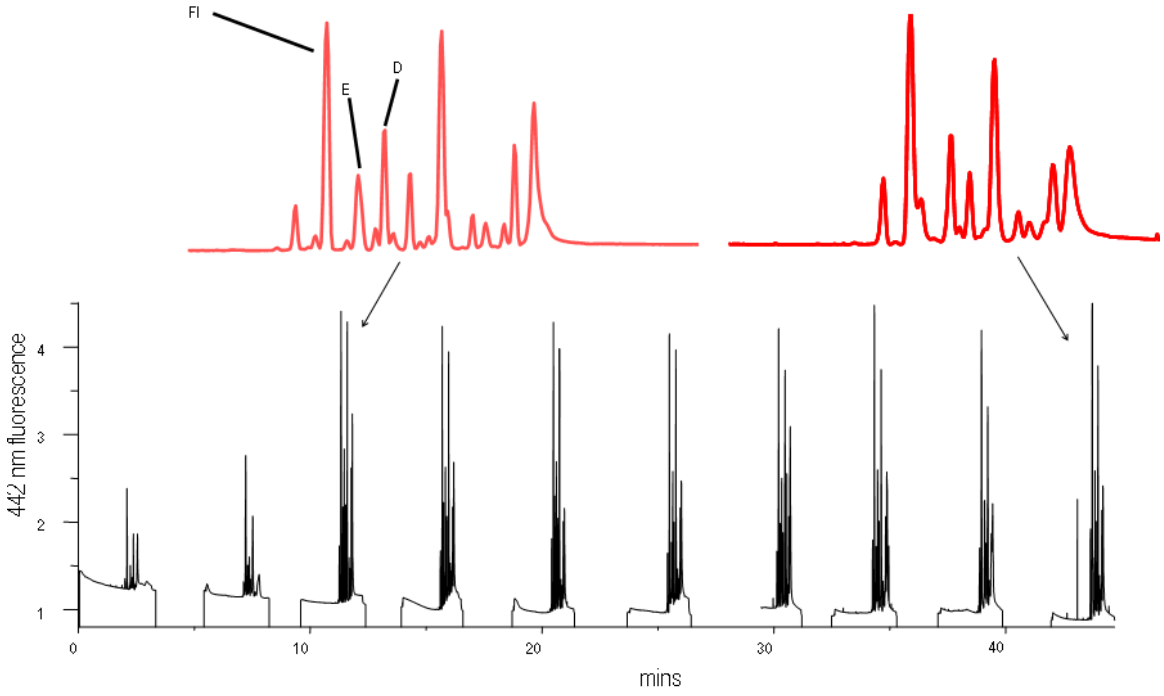

Figure 25: Continuous injections of microdialysis sample of *in vivo* monitoring Glu, Asp and fluorescein under following separation conditions: 200 mM boric acid; 80 mM SDS; 5% acetonitrile; pH 9.2; 400 V/cm. A rat was injected with bolus dose of 0.5 mg fluorescein through the femoral vein.

3.5 Summary

A microdialysis-microchip electrophoresis system was successfully developed by optimizing several key parameters. There were three main achievements from offline studies: (i) a serpentine channel was fabricated in PDMS and characterized (ii) the separation of standards of brain amino acids was optimized and (iii) concentration changes were measured *in vivo* following infusion of high K^+ aCSF. These offline studies aided us in reaching our goal to develop an online continuous *in vivo* monitoring setup. There were several accomplishments from online studies: (i) mixing and reaction of amino acids with NDA and CN^- occurred in the derivatization channel (ii) online *in vivo* monitoring of Glu and Asp was accomplished, and (iii) fluorescein was incorporated into the analysis for future possibilities to perform BBB permeability studies on such systems.

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CHAPTER 4

SUMMARY AND FUTURE DIRECTION

4.1 Summary

In the previous chapter, we have successfully developed a microdialysis-microchip electrophoresis system for online *in vivo* monitoring of Glu and Asp. The PDMS-based system was systematically optimized for separation as well as on-chip derivatization of amino acids with NDA and CN^-. For the first time in our group, proof of concept continuous analysis was performed for excitatory neurotransmitters (Glu and Asp) from the striatum of an anesthetized SD rat. Detection of fluorescein was also incorporated in the system for *in vivo* monitoring of BBB permeability. The ultimate goal is to apply this online system for the investigation of temporal relationship between the release of amino acid neurotransmitters and loss of integrity of BBB during ischemic stroke in the rat model.

4.2 Preliminary studies

4.2.1 Gated injection

Our ultimate goal is to employ this system to monitor dynamic changes in the concentration of amino acid neurotransmitters in awake, freely moving animals. However, in order to detect such fast changes (within 1-5 minutes), temporal resolution of the system needs to be considered. Temporal resolution can be affected by many factors (i) diffusion of analytes from regions distant from the probe to the

probe surface, and (ii) the ability to perform fast repeated injections and separations on chip so that the time-resolved concentration change of analytes can be captured in terms of changes in peak height.

In order to enhance the temporal resolution in a microchip device, the simplest method is to incorporate an electrokinetic gated injection scheme. Some preliminary results using this scheme were obtained on a microchip device that was modified based on a report by Sandlin et. al. [1] ; their work described an electrokinetic gating injection on a serpentine glass microchip. In our experiments, a PDMS chip was fabricated based on this work using standard photolithographic techniques. The injection and separation scheme for this device is shown in Figure 26. It consisted of a sample reservoir (SR) at top from which sample is being electrokinetically pushed by voltage application in the separation channel that simulated hydrodyamic flow from the microdialysis probe for flow-through injection. The sample was injected by floating the buffer reservoir (BR) and applying positive and negative voltages at sample reservoir (SR) and buffer waste (BW) respectively. The size of the injection plug was dependent on the time for which voltage was floated at SR. Immediately after injection, the plug of sample was separated by applying the high positive voltage (5KV) at BR and high negative voltage (-5KV) at BW. A voltage cycle was setup using Labview software following the scheme described above that that resulted in repeated injections and separations without any loss of time in between.

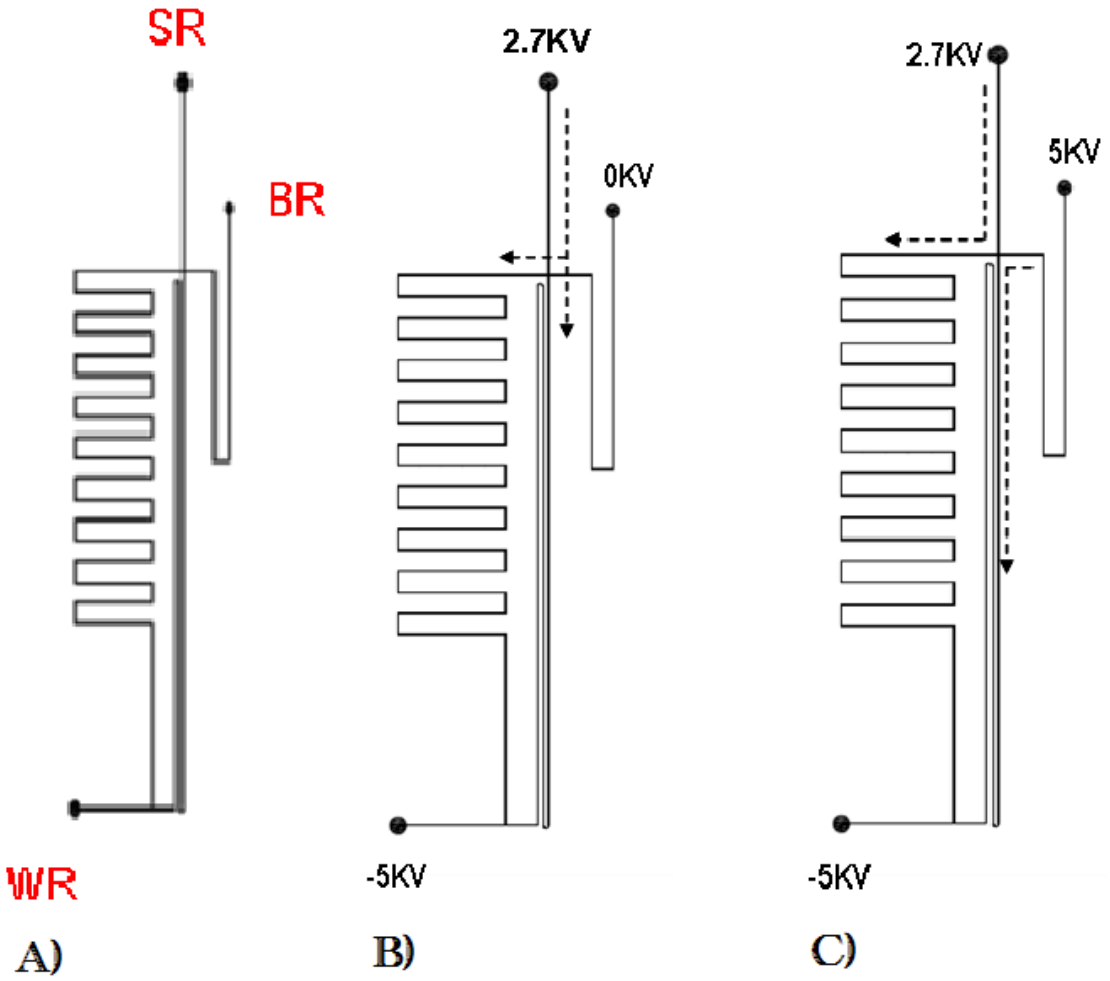

Figure 26: A) Serpentine microchip electrophoresis design for high throughput analysis with electrokinetic gating injection. B) Demonstrates the injection of sample. C) Demonstrates the separation of plug of sample.

Initially, the system was evaluated by continuous injection from 6.2 μM fluorescein solution with the injection scheme described above and shown in Figure 27. The temporal resolution obtained from this experiment was 10 sec with RSD of 4.2% calculated from the peak height. This data supports that high throughput analysis can be accomplished for online *in vivo* monitoring with such a PDMS chip design.

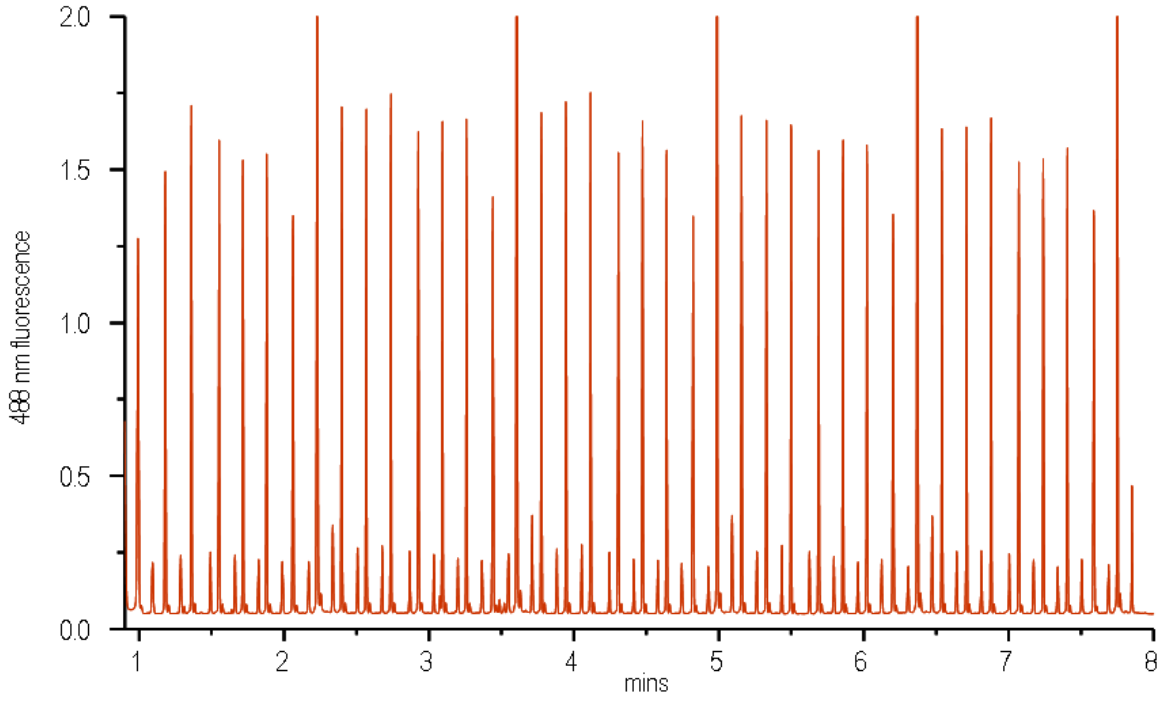

Figure 27: Electropherogram of continuous injection of 6.2 µM fluorescein solution with temporal resolution of 10 sec under following separation conditions: 20 mM boric acid; 10 mM SDS; 10% acetonitrile at pH 9.2.

4.2.2 Improve in mixing

In order to improve mixing of microdialysis sample with NDA and CN^-; a more complicated design was evaluated based on a design by Dolomite Microfluidics [2]. An initial study was performed with three different dyes to investigate mixing. Micrographs were taken at various positions within the reaction channels as shown in Figure 28. The micrograph on bottom right shows that the color becomes homogenous starting from the middle of the reaction channel. Also, to study the degree of mixing for the actual experimental reagents with an amino acid, the fluorescence generated from the derivatization of NDA and CN- with 1mM Glu was monitored within reaction channels as shown in Figure 29. The bright fluorescence observed in the middle of the channels verified that adequate mixing and derivatization was accomplished for this application.

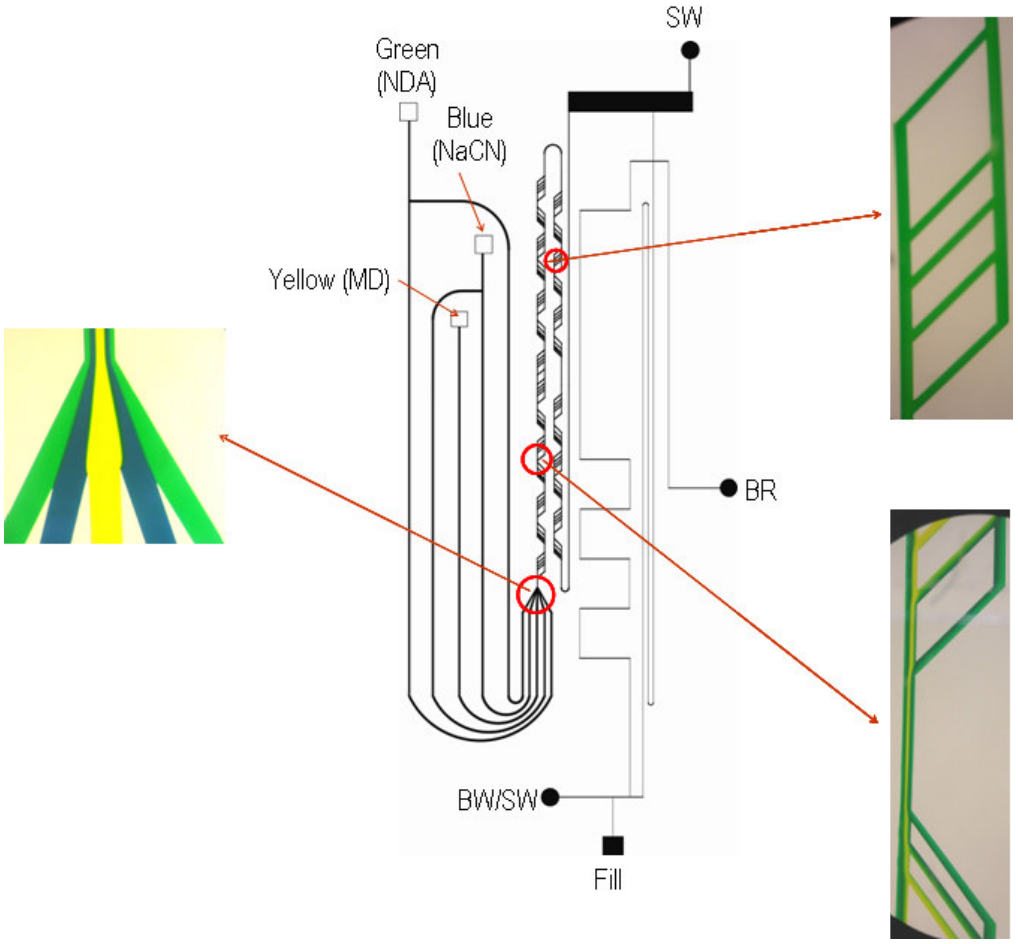

Figure 28: Demonstration of mixing with three different dyes representing NDA, microdialysis sample, and CN^- flows within reaction channels. The rectangular pictures are the micrographs taken at various points within the derivatization channels.

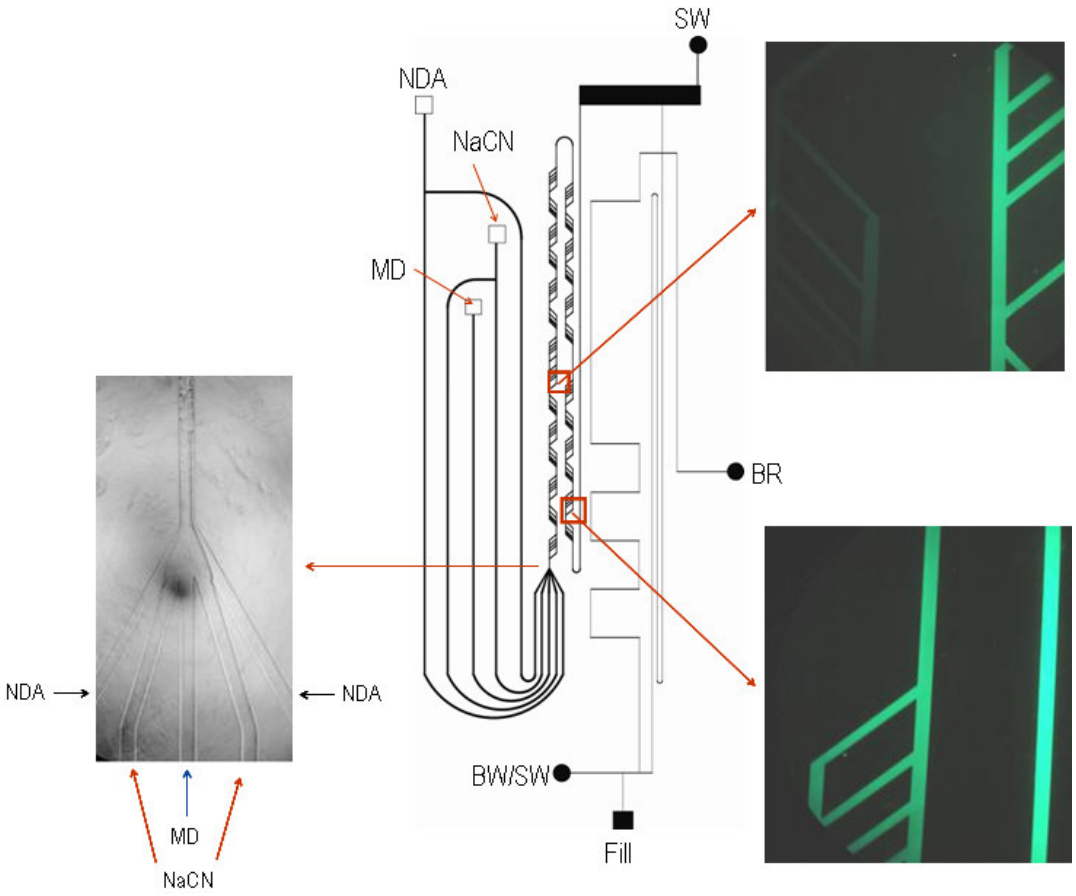

Figure 29: Demonstration of mixing by monitoring the fluorescence from 1 mM Glu derivatized with NDA and CN^-. The square pictures are the micrographs taken at various points within the derivatization channels.

4.3 Future directions

The microdialysis-microchip system developed in this thesis will be a useful device to continuously monitor various amino acids of interest from the rat brain in disease states. In this work, analysis was focused on excitatory neurotransmitters particularly Asp and Glu because their concentration is high in the brain. Although continuous analysis experiments were performed, an online dynamic change in the concentration of Glu and Asp on chip was not demonstrated. Such an experiment will further prove the applicability of the system for monitoring stroke *in vivo*.

In order to improve the reaction of amino acids with NDA and CN^- in the derivatization channel, other nucleophiles as replacement for CN^- is worth investigation as it may substantially improve the speed of the reaction (short derivatization channel) as well as yield better quantum efficiency to achieve high sensitivity on chip. Currently, investigations are underway to explore 2-mercaptoethanesulfonic acid (MESA) as an alternative which has shown promising results with electrochemical detection. However, the stability and fluorescence properties of the products of this reaction still need to be determined.

Further improvement of detection sensitivity is also required to push the detection limits for the analysis of other important endogenous inhibitory neurotransmitter such as γ-amino butyric acid (GABA). The concentration of GABA

in brain sampled by microdialysis has been reported in nanomolar range [3]. Further improvement in detection sensitivity can be achieved by chip detection system modification. This can be achieved with focusing specific wavelength lasers and use of pinhole filters.

A significant drawback of current system is that even though the separation device is miniaturized, the detection system and the accessories that are used to run experiments on chip are mostly of standard size. Therefore, to implement this device for true on-animal monitoring, the power supplies and the detector need to be miniaturized. Such efforts are ongoing in our group for the development of a compact, integrated, electrochemical detector based system for the analysis of nitric oxide metabolite and catecholamines in awake freely roaming sheep.

4.4 References

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